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Host Cell Attachment by Lyme Disease and Relapsing Fever Spirochetes: A Dissertation

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HOST CELL ATTACHMENT BY LYME DISEASE AND RELAPSING FEVER SPIROCHETES

A Dissertation Presented

By

Vivian May Benoit

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Molecular Genetics and Microbiology

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Abstract

Host cell attachment by pathogenic bacteria can play very different roles in the course of infection. The pathogenic spirochetes *Borrelia hermsii* and *Borrelia burgdorferi* sensu lato which cause relapsing fever and Lyme disease, respectively, are transmitted by the bite of infected ticks. After transmission, these spirochetes can cause systemic infection. Relapsing fever spirochetes remain largely in the bloodstream causing febrile episodes, while Lyme disease will often colonize a variety of tissues, such as the heart, joint and nervous system, resulting in a chronic multisystemic disorder. *Borrelia* species have the ability to bind to various cell types, a process which plays a crucial role in pathogenesis and may influence spirochetal clearance from the bloodstream. Colonization of multiple tissues and cell types is likely promoted by the ability to bind to components found in target tissues, and many *B. burgdorferi* adhesins have been shown to promote attachment to a wide variety of cells and extracellular matrix components. Different Lyme disease strains have been shown to preferentially colonize certain tissues, although the basis of this tissue tropism is not well understood. In this study we found that among different Lyme disease strains, allelic variation of the adhesin DbpA contributes to variation in its *in vitro* binding activities raising the possibility that this variation contributes to tissue tropism *in vivo*. In studying *B. hermsii* infection, we found evidence by both histological and fluorescence *in situ* hybridization (FISH) analysis of tissues that indicated that red blood cells were removed by tissue resident macrophages in infected mice. Spirochetes in the spleen and liver were often visualized associated with RBCs, lending support to the hypothesis that direct interaction of *B. hermsii* spirochetes with RBCs leads to clearance of bacteria from the bloodstream. Our findings indicate that host cell attachment play a key role in the establishment of Lyme disease infection, and in contrast contributes to the clearance of relapsing fever infection.

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CHAPTER I

Introduction

Borrelia **species and human disease**

Borrelia species are helical shaped motile bacteria that belong to the family Spirochaetaceae. Although Borreliae are structurally Gram negative with an inner and outer membrane and are classified as such, their outer membranes are rich in lipoproteins and lack lipopolysaccharide (35, 58). Another unique feature of spirochetes is the periplasmic location of their flagella, also known as axial filaments, which confer a spiral shape and are required for their corkscrew-like motility (16). Other human pathogens in the spirochete family include the agents of syphilis (*Treponema pallidum*) and leptospirosis (*Leptospira interrogans*).

Although nearly 300 *Borrelia* species have been identified (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?id=138), only approximately 15 of these are known to be pathogenic in humans. Pathogenic Borreliae, are associated with two important worldwide diseases, Lyme disease and relapsing fever (16). Lyme disease is caused by at least seven species of *Borrelia,* with the most common being *Borrelia burgdorferi* sensu lato, which includes *Borrelia burgdorferi* sensu stricto (herein referred to as *B. burgdorferi*), *Borrelia garinii* and *Borrelia afzelii*. In Europe and Asia, Lyme disease is caused by all three species, while in the United States only *B. burgdorferi* is the major agent of disease (117, 142, 150). Louse-borne relapsing fever is caused by *Borrelia recurrentis*, and is the cause of epidemic outbreaks. Tick-borne relapsing fever, also called endemic relapsing fever, is caused by *Borrelia hermsii*, *Borrelia turicatae*, and *Borrelia duttonii* (50). While not as common as Lyme

disease, relapsing fever is nonetheless an important disease model for antigenic variation and valuable for the study of the immune system (16, 145). Although both Lyme disease and relapsing fever are widely studied, many facets of the pathogenesis of these diseases still elude researchers.

Lyme disease and the biology of *Borrelia burgdorferi*

Lyme disease is a complex, multisystemic disease. First recognized in 1976 in Lyme, Connecticut in a group of children thought to have juvenile rheumatoid arthritis, the cause of the disease was not identified until 1982 (29). It is the most common arthropod borne disease in the United States with nearly 40,000 cases reported in 2009 (http://www.cdc.gov/ncidod/dvbid/lyme/index.htm). Lyme disease occurs in three stages: localized or early stage, disseminated stage, and persistent or chronic stage (for reviews on Lyme disease, see (101, 142)). During the localized stage, seven to ten days after the tick bite, spirochetes colonize the skin and can spread causing the characteristic bull's-eye rash, erythema migrans (EM). Other early symptoms of Lyme disease include fever, malaise, and other flu-like symptoms. During the disseminated stage, which occurs 3 weeks to one month later, spirochetes spread into the bloodstream and multiple second EM lesions can occur. At this time spirochetes may affect the central nervous system causing complications such as meningitis. The chronic stage of Lyme disease can occur months to years after the initial tick bite if left untreated. Lyme disease spirochetes have been shown colonize multiple tissues including the heart, joints, spleen, bladder, and

the nervous system (101). The ability of Lyme disease Borreliae to colonize multiple tissues in the mammalian host can lead to a variety of complications. Carditis, cardiac arrhythmia, and AV block have all been noted in chronic Lyme patients. Arthritis, and muscle inflammation can result from colonization in the musculoskeletal system. Splenomegaly and damage to the liver and heart can occur and are also commonly seen in the murine model of Lyme disease. Bell's palsy, neuroborreliosis, and even psychiatric symptoms have been noted in Lyme disease patients indicating that Lyme disease spirochetes are capable of colonizing multiple tissues in the nervous system as well (71). Chronic Lyme disease complications greatly depend on the species with which one was infected. Most notably, the chronic manifestations commonly seen in Europe differ greatly and from those in North America, and evidence suggests that these differences depend on the *Borrelia* species prevalent in these areas (82, 150).

Certain *Borrelia* species, such as *B. afzelii*, generally do not disseminate but instead cause a local skin infection known as acrodermatitis (acrodermatitis chronica atrophicans), seen in approximately 10% of cases in Europe (141, 146). Other strains do have the ability to disseminate and tend to preferentially colonize certain tissues such as the joints and the nervous systems. For example, *B. garinii* is associated with neurological complications such as facial nerve paralysis (Bell's palsy) (96), while *B. burgdorferi* is associated with Lyme arthritis which is the most common chronic manifestation of Lyme disease in the U.S. (142), and as mentioned above, *B. burgdorferi* is the only *Borrelia* species that has been identified in clinical cases of Lyme disease in the United States (117).

In order to cause disease, *B. burgdorferi* must adapt to two very different environments. In an unfed *Ixodes* tick, *B. burgdorferi* colonizes the tick midgut, and after a bite by an infected tick spirochetes migrate from the midgut to the salivary glands. Due to the relatively slow migration process, ticks must feed for 24-48 hours in order to transmit spirochetes. Several studies have shown that as the tick begins its blood meal, spirochetes begin to alter their gene expression pattern as their migration progresses (135). Certain genes, such as outer surface protein (Osp) A, which is required for *B. burgdorferi* to persist in the tick (168), are downregulated during transmission to the host (48). OspC on the other hand, is upregulated during this time and is required for *B. burgdorferi* infectivity in the mammalian host (65). In addition, *Borrelia* genes that are only expressed in the mammalian host such as OspE, OspF, DbpA and the Elp (OspE-like) proteins have also been identified (66, 109, 127).

Borrelia **genetics and disease severity**

Within a species there can be considerable variation in the ability to cause disseminated disease. However, the genetic basis for the varying ability of these genospecies to cause disseminated disease and preferentially colonize certain tissues is still not known. Several groups have undertaken the task of distinguishing groups of *Borrelia burgdorferi* sensu stricto based on genotype of the highly variable *ospC* allele (53, 136, 158), or by RFLP analysis of rRNA gene spacer types (RST) (91, 92, 155, 156). By comparing different isolates of *B. burgdorferi* from the skin, blood and CSF, it was shown that different strains of *B. burgdorferi* can be subdivided into 21 different groups

according to the sequence of their *ospC* alleles. Based on *ospC* lineage, it was shown that certain strains were more invasive while others tended not disseminate and caused a localized skin infection only (53). An analogous pattern could be found when different strains were grouped by RST, where certain genotypes occurred at a greater frequency in strains isolated from the blood of infected patients than those isolated from skin (91, 92).

Genetic studies of *Borrelia* species have greatly lagged behind those of other pathogenic organisms. *In vitro* cultivation of this organism is difficult; with a doubling time of > 8 hours and the requirement of a rich defined medium (14). In addition, Lyme disease Borreliae can be sensitive to minor changes in medium formulations, requiring careful attention to preparation and batch-to-batch variation (154). It was not until 1997 that the genome sequence of one strain of *Borrelia burgdorferi* (strain B31) was completed (58). The availability of this sequence revealed several important features of *Borrelia* species, including the small size and complexity of its genome (~1.5 Mb) and the presence of extrachomosomal linear and circular plasmids, which can vary in number between Lyme disease *Borrelia* species (34, 35). The sporadic loss of plasmids during *in vitro* growth greatly complicates our ability to generate defined genetic lesions in this organism, thus hampering genetic analysis of virulence (10, 11, 80, 105, 120, 140). In addition, there are at least two known endogenous plasmid encoded restriction-modification systems which rapidly degrade exogenous DNA, further hampering genetic manipulation of *Borrelia* species (81).

Within the last ten years, however, several tools have been developed that greatly advanced the ability of researchers to perform genetic studies with *B. burgdorferi*. These

tools include the development of shuttle vectors (144), the development of methods for mutagenesis including transposon mutagenesis (143, 169) and modification of the Cre-lox recombination system for use in *B. burgdorferi* (19). In addition, our lab has shown that *in vitro* CpG methylation protects DNA from degradation and greatly enhances the efficiency of *Borrelia* transformation (37). Purser *et al*. showed linear plasmid (lp) 25 harbors the gene *pncA* (BBE22), which encodes a nicotinamidase and is required for infectivity in mice. By complementing a strain lacking lp25 with a shuttle vector containing BBE22, the authors were able to restore infectivity in the murine host to near wild-type levels (119). Plasmid lp28-1 has also been shown to be required for infectivity in the mouse model (120), as well as being associated with causing Lyme related arthritis (165). Within these plasmids, several genes which are required for cell adhesion and infectivity such as *dbpA* and *dbpB* (encoded on lp54) (67, 159), *ospC* (encoded on cp26) (128), and *vlsE* (encoded on lp28-1) (172) have also been identified.

Cell adhesion by Lyme disease spirochetes

Host cell attachment is thought to be a critical first step for many pathogens for the establishment of infection. Cell adhesion by *B. burgdorferi* has been widely studied, and it has been shown that this spirochete is able to bind to multiple different cell types *in vitro* including epithelial, endothelial and glial cells, platelets and lymphocytes (45, 49, 59, 60, 147, 148). Lyme disease spirochetes can invade and colonize multiple tissues in the untreated host, and have been shown to bind to multiple different host cell and extracellular matrix components such as integrins (39), fibronectin (24, 64, 118),

proteoglycans (67, 78, 86), laminin (25, 152), and collagen (171). A notable feature of Lyme disease Borreliae is that these pathogens express multiple different adhesins, each of which bind to one or more of these molecules (Table 1-1). In addition, many borrelial adhesins are also required for infectivity, indicating that host cell attachment is indeed crucial for Lyme disease pathogenesis.

Adhesin (gene designation)	Host recpetor	Reference
BBK32 (bbk32)	Fibronectin	Probert and Johnson. 1998, Mol. Micro. 30:1003-1015.
P66 (bb0603)	Integrin aIIbb3 Integrin avb3	Coburn *et al*. 1993, PNAS 90: 7059-7063.
DbpA (bba24)	Decorin Dermatan-SO_4	Guo *et al*. 1998, Mol. Micro 30: 711-723.
DbpB (bba25)	Decorin Dermatan-SO_4 Chondroitn-6-SO_4	Guo *et al*. 1998, Mol. Micro 30: 711-723.
Bgp (bb0588)	Heparan-SO_4 Dermatan-SO_4	Parveen and Leong. 2000, Mol. Micro. 35: 1220-1234.
OspA (bba15)	TROSPA (tick receptor for OspA	Pal *et al*. 2004, Cell 119:457-468.
RevA (bbM27)	Fibronectin	Brissette *et al*. 2009, I&I . 77:2802-2812.
Bmp family (bb0382-0384)	Laminin	Verma *et al*. 2009, I&I 77: 4940-4946.

Two of the most widely studied of the *Borrelia* adhesins are decorin binding proteins (dbp) A and B. DbpA and B are highly related surface lipoproteins first identified by showing that radiolabeled *B. burgdorferi* bound to decorin (67). These proteins are encoded on a single operon and each bind to decorin, a proteoglycan with a dermatan sulfate glycosaminoglycan (GAG) side chain that "decorates" collagen fibers in mammalian tissues (66, 67, 70). To establish an infection, Lyme disease spirochetes must migrate from the tick midgut to the salivary glands, and from there encounter the skin of the mammalian host. In order to cause disseminated infection spirochetes

colonize the skin, a tissue rich in collagen. Although some *Borrelia* strains bind directly to collagen depleted of decorin *in vitro* (171), this study did not rule out a requirement for decorin in collagen binding *in vivo*. Some studies have shown that DbpA and DbpB require both the protein core and the GAG side chain for full binding to decorin (26, 115) while other studies have shown that the GAG side chain alone is sufficient for binding (57).

Lyme disease spirochetes have been shown to recognize distinct classes of GAGs, and variations in GAG-binding specificity appear to lead to differences in the types of mammalian cells recognized (113). For example, *B. burgdorferi* strain N40, which recognizes heparan sulfate and dermatan sulfate GAGs, binds efficiently to glial and endothelial cells *in vitro*, while *B. afzelii* strain VS461, which predominately recognizes dermatan sulfate, binds selectively to glial cells (113). In addition to binding decorin, DbpA and B from *B. burgdorferi* strain N40 differ in their GAG and mammalian cell type binding specificity (57). Besides DbpA and B, *B. burgdorferi* encodes other GAG binding proteins, such as Bgp and BBK32 (56, 111). These findings, together with the fact that Lyme disease spirochetes encode multiple GAG binding adhesins, suggest an important role for GAG binding in Lyme disease pathogenesis, and that strain specific differences in GAG recognition by Lyme disease spirochetes might influence the specificity of host cell attachment and thus tissue colonization during infection.

Glycosaminoglycans are a family of carbohydrates that consist of linear repeating disaccharide (a hexose or hexuronic acid and a hexosamine) chains. Composition of

GAGs can vary in a number of ways including the type sugar, glycosidic linkage, acetylation, and sulfation pattern. In addition to differences in structure, GAGs can also be either soluble or membrane bound. GAGs are found in a variety of mammalian tissues including the extracellular matrix, cartilage, and connective tissues. For example, heparan sulfate is the most ubiquitous GAG with 50-90% of total endothelial proteoglycans consisting of this polysaccharide. In contrast, dermatan sulfate is found mainly on cell surfaces and in the extracellular matrix and is the predominant GAG found in the skin, while chondroitin sulfates are the major GAG species found in the central nervous system (72, 90, 149). A number of different biological processes involve GAGs, including cell adhesion, receptor engagement and signal transduction (54, 79, 87). As mentioned above, Lyme disease spirochetes have been shown to colonize multiple different tissues in very diverse hosts which can vary in their quantity and composition of GAGs. Due to the structural diversity of GAGs, and the ubiquitous nature of these carbohydrates, one can imagine that Lyme disease Borreliae have evolved different GAG-binding adhesins in order to facilitate movement from the bloodstream to multiple different tissues during infection.

DbpA and B play crucial roles in Lyme disease pathogenesis. Decorin was initially implicated in Lyme disease pathogenesis in experimental infections with decorin knockout ($Dcn^{-/-}$) mice (27). This study showed that in low dose infection, $Dcn^{-/-}$ mice had a lower incidence of dissemination to the joint and heart, a reduced incidence and severity of arthritis compared to $Dcn^{+/-}$ and $Dcn^{+/+}$ mice. This study was the first to show that decorin binding may have a role in spirochete dissemination and colonization of

certain tissues, as well requiring an interaction with decorin to cause arthritis. Shi *et al*. initially reported that DbpA and DbpB are not required for infectivity in the mouse model (138). This study examined a single high dose (10^5) infection in immunocompetent mice and SCID mice. While there was no difference in tissue colonization in SCID mice, there was a mild defect in colonization of the joint (11/16 culture positive) and a more severe defect in colonization of the heart (3/16 culture positive) in immunocompetent mice (138), in agreement with the study using *Dcn*$^{-/-}$ mice which showed the same colonization defect. The defect in colonization in the joint and heart was attributed to a possible defect due to genetic manipulation of their mutant, although a complemented mutant was not generated and analyzed. However, a later study more comprehensive study by Shi *et al*. showed that while inactivation of the *dbpBA* locus did not completely abolish infectivity, the DbpA and B proteins are required for full virulence in mice infected by needle inoculation (137). By deleting the *dbpBA* operon from strain B31 and examining multiple infectious doses in the mouse model, Weening *et al*. showed that DbpA and B are required for initial colonization and persistence in the murine host. Additionally, this study showed that DbpA and B are required for dissemination to, and colonization of certain tissues (159). Liang and colleagues showed that overproduction of DbpA in an *ospC* deficient mutant reduces the ID_{50} and protects the bacteria from immune clearance in the skin of SCID mice. This study also revealed that overproducing DbpA impairs dissemination from the initial site of infection (166). Blevins *et al*. reported that DbpA and B are required for virulence in mice via needle inoculation, but not via tick inoculation (21), consistent with the fact DbpA and B are not expressed by *Borrelia* in

the tick vector (36, 167). These studies suggest that DbpA may have several different roles in experimental infection, including key roles in dissemination and tissue colonization in the mouse model.

DbpA is highly polymorphic among Lyme disease spirochete strains while DbpB is highly conserved (123). In addition, recombinant derivatives of DbpA allelic variants differ in decorin binding (115). However, the role of allelic variation of DbpA has not previously been examined. The apparent requirement for DbpA in experimental Lyme disease pathogenesis and the diversity of *dbpA* alleles in different Lyme disease Borreliae, lead us to examine whether DbpA from multiple species influences binding to specific cell and tissue types and what role this variation may play in pathogenicity of Lyme disease.

Relapsing fever

Relapsing fever is a disease that has been recorded since ancient times and is caused by the bite of infected soft-bodied (*Ornithodorus*) ticks or by exposure to the body louse (*Pediculus humanus*) (50). Mice and other small rodents are the natural reservoirs for relapsing fever spirochetes. This group includes *B. recurrentis*- the cause of epidemic outbreaks; while *B. hermsii*, *B. duttonii*, and *B. turicatae* are present in endemic areas. The most prevalent strain in the United States is *B. hermsii*, although less than 500 cases have been reported since 1977 (50). Relaspsing fever spirochetes persistently colonize the salivary glands of *Ornithodorus* ticks. Thus, in contrast to *Ixodes* ticks which must

feed for 24-48 hours in order transmit Lyme disease, *Ornithodorus* ticks require only 15-90 minutes to transmit relapsing fever (134). After an incubation period of a few days to a few weeks, spirochetes spread from the site of infection to the blood and the infected host experiences recurring episodes of high fever lasting several days followed by an afebrile period. Without treatment these episodes can last for several months, with up to thirteen relapses and the duration of the afebrile period increasing between each occurrence of fever (for a recent review, see (51)). The relapse phenomenon has been widely studied, and is now known to be caused by a switch in the major surface antigen expressed by relapsing fever spirochetes while in the mammalian host.

Antigenic variation

Studies have shown that the febrile period coincides with a peak in bacteremia ($\sim 10^9$/ml) in the blood (29), while the afebrile period is due to an expansion of B1b lymphocytes and anti-*Borrelia* (IgM) antibodies in the blood (2). In order to evade the immune system, relapsing fever *Borrelia* species undergo switching of their surface antigens, called Vsp's (variable small proteins) and Vlp's (variable large proteins) (I will collectively refer to these proteins by their former name, Vmp's- variable major proteins), each a unique serotype (74). It was recognized early in the twentieth century that relapsing fever *Borreliae* undergo antigenic variation, although the idea that relapses were due to the outgrowth of antigenically distinct populations of *Borrelia* was not widely accepted at the time (133). This phenomenon was nonetheless recognized as an important tool for studying the immune system (145). In 1967, Coffey and Eveland

developed a method to identify specific populations of spirochetes obtained in a rat model of relapsing fever, and showed that spirochetes obtained from relapses were in fact of distinct serotypes. In addition, the authors also found evidence that the serotype switching occurred in a predictable sequence (41, 42). In a later study, Stoenner *et al*. provided evidence that as many as 25 unique serotypes can be generated from a single serotype, indicating that antigenic variation of relapsing fever spirochetes was far more complex than originally thought (145). In addition, Stoenner showed that it was possible to recover the original serotype by infecting a naïve mouse with spirochetes recovered from a relapse population. These findings were important because the data indicated that serotypes were not lost, and that antigenic variation is reversible (145).

The Vmp genes are encoded on linear plasmids, and while in the tick vector *B. hermsii* expresses Vsp33 (sometimes called Vtp33) (134). Once a mammalian host is infected, a different Vmp is expressed. Various studies revealed that the mechanism of antigenic variation involves gene conversion between a silent Vmp on an archive plasmid and the current Vmp on the expression plasmid (15). For example, if Vsp21 is currently expressed and then replaced by Vsp7 at the expression locus, the new predominant serotype will be Vsp7. Since there is very little cross-reactivity between the different serotypes, antibodies developed against the formerly expressed Vmp are ineffective against the newly expressed antigen thus allowing outgrowth of spirochetes during the relapse (9, 145). Although the host immune response and mechanism of immune evasion by *B. hermsii* and other species of relapsing fever spirochetes is well studied, little is known about how this pathogen is cleared from the infected host.

Host cell binding by relapsing fever *Borrelia* species

Approximately 25 cases of relapsing fever are reported per year in the United States, although cases may be under reported since symptoms mimic other illnesses (http://www.cdc.gov/ncidod/dvbid/relapsingfever/index.htm). Due the lack of mortality in cases of relapsing fever and the rare occurrence of the disease, very little is known about its pathogenesis. In addition to episodes of high fever due to bacteremia, hallmark clinical features of relapsing fever include anemia and thrombocytopenia. It is currently unknown how *B. hermsii* causes these hematological changes. Various Lyme disease spirochetes have been show to bind integrin $\alpha_{IIb}\beta_3$, although this capability appears to be limited to infectious strains (38, 40). Likewise, *Borrelia hermsii* also bind to platelets. This interaction results in platelet and $\alpha_{IIb}\beta_3$ activation, which in turn enhances binding by spirochetes (5, 39). Although *B. hermsii* activates platelets, an additional study showed that spirochete-bound platelets in the bloodstream are in the resting state. This study also showed that platelet binding by *B. hermsii* can also occur independent of $\alpha_{IIb}\beta_3$ and that this integrin is not involved in the induction of thrombocytopenia (6). In relapsing fever patients, it is currently not known what becomes of activated platelets once they are bound by spirochetes, or how this interaction may contribute to thrombocytopenia.

In blood smears of infected mice, *B. hermsii* can be seen by darkfield microscopy bound to red blood cells (RBCs) as well as to platelets (68). Another relapsing fever spirochete, *B. crocidurae*, becomes coated with red blood cells while in the bloodstream,

a phenomenon known as erythrocyte rosetting (30). As with platelet binding by relapsing fever spirochetes, it is also unknown whether spirochete binding to RBCs is indeed the cause of anemia seen in human patients as well as in experimental infection. In our study, we initially set out to test whether genetic background of the infected host plays a role in the outcome of relapsing fever infection. In addition to our findings regarding the influences of host genetics and immune response to *B. hermsii* infection, we found that *B. hermsii* appears to invade the heart, spleen and liver of infected mice, and associates with RBCs within these tissues. These observations lead us to hypothesize that RBC binding by *B. hermsii* may be a mechanism that leads to clearance of bacteria from the bloodstream- a sharp contrast to *B. burgdorferi* which appears to use host cell binding to establish its infection.

Summary

In this study, we used the murine models of relapsing fever and Lyme disease in order to study the role of host cell attachment in the pathogenesis of these two important human diseases. In the relapsing fever model, we analyzed the role of genetic background of the host in resistance to this disease. While studies have been previously conducted to identify mammalian genes which influence sensitivity to *B. burgdorferi* infection (124, 160), this study was the first of its kind to examine the role of genetic background of the host in the control of *B. hermsii* infection. We found profound differences in the outcome of *B. hermsii* infection in mice which indicate that the innate

immune response to this infection is under genetic control. It is currently unknown how *B. hermsii* infection causes anemia or thrombocytopenia. We found that host cell attachment may play a role in the clearance of this pathogen via binding to RBCs, an interaction that has previously been implicated as having a role in this disease. In the Lyme disease model, we examined how allelic variation of a specific adhesin, DbpA, influences host cell binding and whether or not the heterogeneity of this surface located protein may be responsible for the tissue tropism seen in different Lyme disease genospecies. Lyme disease spirochetes can colonize multiple tissues in the mammalian host, and tissue colonization appears to be strain- and species-specific. We used a non-adherent *B. burgdorferi* strain to characterize the binding phenotypes of different *dbpA* alleles. We found that allelic variation of DbpA leads to dramatic differences in the ability of this adhesin to promote bacterial attachment to GAGs and mammalian cells. Using these two murine infection models, we were able to show that host cell attachment can play very different roles in bacterial pathogenesis.

CHAPTER II

Genetic control of the innate immune response to *Borrelia hermsii* influences the course of relapsing fever in inbred strains of mice

Abstract

Host susceptibility to infection is controlled in large measure by the genetic makeup of the host. Spirochetes of the genus *Borrelia* include nearly 40 species of vector-borne spirochetes that are capable of infecting a wide range of mammalian hosts, causing Lyme disease and relapsing fever. Relapsing fever is associated with high-level bacteremia, as well as hematologic manifestations such as thrombocytopenia (i.e. low platelet numbers) and anemia. To facilitate studies of genetic control of susceptibility to *Borrelia hermsii* infection we performed a systematic analysis of the course of infection using immunocompetent and immunocompromised inbred strains of mice. Our analysis revealed that sensitivity to *B. hermsii* infections is genetically controlled. In addition, whereas the role of adaptive immunity to relapsing fever spirochetes is well documented, we found that innate immunity contributes significantly to reduction of bacterial burden. Similar to human infection, progression of the disease in mice was associated with thrombocytopenia and anemia. Histological and fluorescence in situ hybridization (FISH) analysis of infected tissues indicated that red blood cells were removed by tissue resident macrophages, a process that could lead to anemia. Spirochetes in the spleen and liver were often visualized associated with RBCs, lending support to the hypothesis that direct interaction of *B. hermsii* spirochetes with RBCs leads to clearance of bacteria from the bloodstream by tissue phagocytes.

Introduction

The *Borrelia* genus is formed by a group of bacteria that are small flexible helical spirochetes. Lyme disease spirochetes, *B.burgdorferi sensu lato* (114), cause the most common arthropod-borne illness in the U.S. and are responsible for more than 20,000 reported cases per year (117). Relapsing fever is another important worldwide infection that is caused by several species of the genus *Borrelia* and is typified by high levels of growth of spirochetes in the bloodstream. Epidemic relapsing fever is caused by *Borrelia recurrentis* and transmitted by body lice (47). Endemic relapsing fever is transmitted by ticks and caused by several species of *Borrelia*, such as *B. hermsii*, *B. turicatae*, and *B. duttoni (52)*).

B. hermsii is the most important relapsing fever spirochete in the United States and is acquired by the bite of an infected *Ornithodorus hermsi* tick (50). The first manifestation of relapsing fever is associated with high-titer (10^6 to 10^8 spirochetes/ml) growth of the spirochete in blood. This infection is typified by recurrent febrile episodes, each of which corresponds to high-level bacteremia caused by antigenically distinct populations of bacteria (reviewed in references (9, 12, 135). Antigenic switching is a consequence of the sequential expression of genes for serotype-specific major surface antigens known collectively as variable major proteins (Vmps). The mechanism of antigenic variation involves a gene rearrangement to localize a new variant *vmp* gene at a unique expression site (17). The spirochete encodes many silent *vmp* genes, and at least 25 antigenically distinct serotypes of this bacterium can be generated from a single

bacterium (122, 145). Thus, a cycle of bacteremia, clearance, and outgrowth of antigenic variants can occur several times, giving rise to a relapsing illness. The high-titer growth of *Borrelia* in the bloodstream results in a wide range of symptoms that include fever, chills, and muscle and joint aches. After 2 to 9 days, these symptoms disappear, corresponding to the first clearance of bacteria from the blood, but the recurring nature of the bacteremia results in the reappearance of symptoms for several weeks, if left untreated. In addition, blood infection is associated with several striking hematological abnormalities. For example, thrombocytopenia, i.e., a low platelet count, is the most frequent laboratory manifestation of *B. hermsii* infection in humans, and normocytic anemia and leukocytosis are also common (22). Both of these manifestations might involve interactions between host cells and blood-borne bacteria, because *B. hermsii* has been demonstrated to bind to platelets and red blood cells (RBCs) (6, 68). In particular, not only were episodes of thrombocytopenia temporally and quantitatively correlated with episodes of bacteremia, but platelet-bacterium complexes were detected in infected mice (4).

Rodents are both natural hosts for relapsing fever spirochetes and provide an experimental model in which to investigate the pathogenesis of human infection. Murine infection recapitulates a number of pathophysiological aspects of the human disease, most notably the hallmark of recurrent episodes of severe (~10^7 to 10^8/ml) bacteremia. In addition, hematological manifestations, such as leukocytosis, anemia, and thrombocytopenia that are commonly observed in human patients are associated with the episodes of recurrent spirochetemia in mice. Thus, the groundbreaking discovery that

immune evasion by relapsing fever spirochetes was due to antigenic variation resulting from genomic rearrangements relied upon murine infection to generate antigenic variants (9, 145). The murine model also provided a system in which to identify critical components of an adaptive immune response required for clearance of *B. hermsii* from the bloodstream. $\mu MT^{-/-}$ mice that lack B cells are completely susceptible to relapsing fever (46), and passive transfer experiments demonstrated that antibodies are essential effector molecules in protection from relapsing fever (7, 170). Interestingly, the B cells confer protection independently from T-cell help (2, 13, 102). T-cell-independent antibody responses occur with remarkable speed, consistent with the observation that each episode of spirochetemia lasts only two or three days. Finally, immune reconstitution of recombination-activating gene (*rag*)-deficient mice revealed that a subclass of T-independent B cells termed B1b cells was capable of conferring long-term immunity to *B. hermsii* infection (3).

Whereas the role of an adaptive immune response in the clearance of relapsing fever spirochetes has been the focus of significant scientific investigation, very little is known about the role of innate immune mechanisms and spirochetemia. Although mice deficient in adaptive immunity, such as *rag* and *scid* mutants, cannot resolve *Borrelia* infection completely, they possess some means of transiently controlling relapsing fever spirochetes. Thus, while infection of $rag2^{-/-}$ mice with *B. hermsii* leads to a rapid increase in the number of blood-borne bacteria that peaks at day 3 postinfection, in the ensuing days, the level of bacteremia in these mice diminishes significantly, indicating that innate immunity contributes to effective control of this bacterial infection. These

observations prompt the question of how mice manage to control bacteremia even in the absence of an adaptive response.

Inbred strains of mice have been used extensively in the analysis of genetic control of infectious diseases (28). Several genes identified by genetic mapping have been shown to play an important role in the development and progression of human diseases. One of the most prominent examples is *Nramp1*/SLC11A1, which encodes a divalent cation transporter (20). *Nramp1* was identified as a gene controlling susceptibility to a wide range of pathogens, such as *Leishmania*, *Salmonella*, and *Mycobacterium bovis* (153). Inbred strains of mice were used extensively for studies of genetic control of differences in sensitivity to diseases induced by *B. burgdorferi* infection. These studies identified several *q*uantitative *t*rait *l*oci (QTL) that control *B. burgdorferi*-induced arthritis and levels of specific and total IgGs (124, 160). Subsequent analysis of 10 different phenotypic readouts identified up to 14 QTL that control various aspects of *B. burgdorferi* infection (124). To date, there are no studies of the contribution of genetic variation of the host to control of *B. hermsii* infection. In order to develop an experimental system to study the role of innate immunity in the control of blood-borne bacteremia, we performed a survey of common inbred mouse strains. We identified several significant differences in the course and outcome of *B. hermsii* infection in inbred strains of mice that indicate genetic control of both innate and adaptive response to this infection.

Experimental procedures

Mice. The mouse strains BALB/cByJ, C57BL/6ByJ, C3H/HeJ, DBA/1J, and SJL/J were purchased from Jackson Laboratories (Bar Harbor, ME). Information regarding the differences in the genetic backgrounds of these mouse strains is available on the Jackson website (http://www.informatics.jax.org/strains_SNPs.shtml). All wild-type mice were females between 4 and 6 weeks of age. Female *rag2*$^{-/-}$ mice in the BALB/c, C57BL/6, and C3H/HeN backgrounds were purchased from Taconic Farms (Germantown, NY). *rag2*$^{-/-}$ mice were also between 4 and 6 weeks of age. Additional male and female *rag2*$^{-/-}$ mice for this study were bred in the animal facility at the University of Massachusetts Medical School. Mice used in this study were housed in microisolator cages in the Department of Animal Medicine.

***B. hermsii* infections and blood sampling.** To generate standardized stocks of highly infectious bacteria, *B. hermsii* bacteria were harvested from the blood of infected secretory IgM-deficient (sIgM$^{-/-}$) mice, which suffer high-level (~10^8/ml) bacteremia, by cardiac puncture and frozen in 20% glycerol until needed. Five *rag2*$^{-/-}$ mice of BALB/c, C57BL/6, or C3H/HeN background were infected intravenously with 10^5 *Borrelia hermsii* strain DAH bacteria (74) in 100 μl of BSK-H medium (Sigma-Aldrich Co., St. Louis, MO). Blood sampling was carried out as previously described (5). Briefly, less than 1 mm of the tail was cut with surgical scissors, and 5 μl of blood was taken with a micropipette and placed in 45 μl of anticoagulant (100 mM citric acid, 100 mM sodium

citrate in phosphate buffered saline [PBS], pH 7.0). Bacteremia was monitored at 24-h intervals for 5 days for wild-type mice and at 12- or 24-h intervals for 14 days for *rag2*$^{-/-}$ mice. The levels of *B. hermsii* bacteria in these blood samples were evaluated by diluting 10 μl of the above-described blood sample into 40 μl of phosphate buffered dextrose (2% dextrose in PBS, pH 7.0) for a final dilution of 1:50. Five-microliter amounts of the diluted blood samples were examined under dark-field microscopy (400X magnification) to quantify the number of *B. hermsii* bacteria, as described previously (5). The limit of detection of *B. hermsii* bacteria by this approach is 1 X 10^5 bacteria/ml.

Flow cytometry. One microliter of whole blood was diluted 1:10 in citrate anticoagulant. Phycoerythrin (PE)-Cy5-conjugated anti-mouse TER-119 (eBioscience) was diluted 1:10, and PE-conjugated anti-mouse CD61 antibody (BD Pharmingen) was diluted 1:200 in phosphate-buffered dextrose to label red blood cells and platelets, respectively. To measure the platelet and red blood cell counts by flow cytometry, 10 μl of SPHERO rainbow fluorescent polystyrene beads (Spherotech, Inc., Libertyville, IL) was added as an internal standard, as described previously (4). The numbers of red blood cells and platelets were measured by flow cytometry using a Becton-Dickinson FACSCalibur. All flow cytometry experiments were performed within 2 days of collection of blood samples.

Histology. Tissues were harvested from infected mice at days 5 and 14 postinfection, fixed in 10% histological-grade buffered formalin, and then embedded in paraffin. Five-

micrometer sections of spleen and liver were cut and then stained with hematoxylin and eosin (H&E).

FISH. For fluorescence *in situ* hybridization (FISH) analysis, tissues were harvested from infected mice at days 5 and 14 postinfection and fixed in 3.7% histological grade formaldehyde with 50% ethanol in phosphate-buffered saline. Tissues were embedded in methacrylate and sectioned as described previously (100). A *Borrelia* genus-specific oligonucleotide probe, reBorr0 (5'-GCATGCTTAAGACGCACTGCC-3'), was designed and 5' end labeled with Cy3 (indocarbocyanine) (Biomers, Ulm, Germany). To control for specificity of the probe, *Borrelia garinii* strain 1B29 (kindly provided by A. Schönberg, Federal Institute for Health Protection of Consumers and Veterinary Medicine, Berlin, Germany) and *Treponema denticola* (ATCC 35405) with two mismatches at the probe binding site were included as positive and negative controls, respectively, in each FISH experiment. Sections (3 μm) were hybridized as published previously (131), using a hybridization buffer containing 20% formamide. After incubation at 50°C for 2 h, slides were rinsed with double-distilled water, air dried, and mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA) containing the nonspecific nucleic acid stain DAPI (4',6'-diamidino-2-phenylindole). For microscopy, an epifluorescence microscope (Axioplan2; Carl Zeiss, Jena, Germany) equipped with narrow-band filter sets (AHF; Analysentechnik, Tübingen, Germany) was used.

Statistical analysis. Data were analyzed using GraphPad Prism. Comparison of multiple groups was performed using one-way analysis of variance (ANOVA), and the significance of differences was evaluated with Bonferroni's multiple comparison test. Statistical significance of difference between two groups was evaluated using two-tailed unpaired *t* tests. In all tests, *P* values below 0.05 were considered statistically significant. In all graphs, error bars represent standard deviations.

Results

Immunocompetent BALB/c mice are relatively resistant to *B. hermsii* infection.

In order to test whether a phenotypic difference in resistance to *B. hermsii* exists among inbred strains of mice, we infected intraperitoneally (i.p.) mice of five different strains, BALB/cByJ, C57BL/6ByJ, C3H/HeJ, DBA/1J, and SJL/J (Jackson Laboratory, Bar Harbor, ME), with 10^5 *B. hermsii* strain DAH bacteria. These strains were chosen to match the progenitors of existing recombinant inbred mapping panels. For this initial survey, female mice were infected in groups of five, and bacteremia was evaluated daily by dark-field microscopy of diluted blood samples as previously described (5). Following the initial experiments, we established that an intravenous (i.v.) route of infection provides a more reproducible course of infection than i.p. injection (data not shown). Thus, all subsequent experiments used i.v. infection for delivery of spirochetes to the bloodstream. Previous extensive study of infections in C57BL/6 mice indicated that peak bacteremia of *B. hermsii* strain DAH infection occurs on day 3 postinfection (3, 6). Thus, to discern potential phenotypic differences among different mouse strains, we chose to compare bacteremia at day 3 postinfection. We found that BALB/cByJ was the most resistant strain, displaying a level of bacteremia 5-fold lower than in C57BL/6ByJ mice and 4-fold lower than in C3H/HeJ mice (Fig. 2-1). DBA/1J and SJL/J mice had intermediate levels of bacteremia (data not shown). Two additional surveys with strains BALB/cByJ, C57BL/6ByJ, and C3H/HeJ revealed these phenotypic differences to be reproducible and significant between BALB/cByJ and C57BL/6ByJ ($P < 0.01$) and

between BALB/cByJ and C3H/HeJ ($P < 0.05$) mice (Fig. 2-1, day 3).

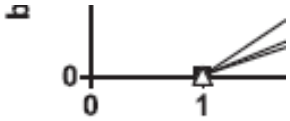

Figure 2-1. Differences in control of *B. hermsii* infection among common inbred strains of mice. Wild-type female BALB/cByJ, C57BL/6ByJ, and C3H/HeJ mice were infected i.v. in groups of five with 105 *B. hermsii* strain DAH bacteria. Bacteremia was monitored daily by microscopic examination of blood smears. The limit of detection of spirochetes by this approach is 1 X 10^5 bacteria/ml of blood. Inset shows spirochete numbers in the bloodstreams of individual mice at day 3postinfection, along with means and standard deviations for the groups. At this time point, there were significant differences in levels of bacteremia between BALB/cByJ and C57BL/6ByJ mice ($P < 0.01$) and between BALB/cByJ and C3H/HeJ mice ($P < 0.05$) as determined by one-way ANOVA with Bonferroni's multiple comparison test.

C57BL/6 *rag2*$^{-/-}$ mice are relatively resistant to *B. hermsii* infection.

Initial observations in *rag1*$^{-/-}$ mice indicated that bacteremia peaked at day 3 or 4 postinfection, followed by a 10- to 20-fold decrease in bacterial load (2). Thus, some degree of control of bacteremia was evident even in the absence of the adaptive immune system, suggesting a substantial contribution of innate immunity in this process. To determine if differences in the response to *B. hermsii* infection observed with the immunocompetent wild-type mice described above might reflect genetic differences in the function(s) of the innate immune system, we analyzed the course and outcome of *B.*

hermsii infection in *rag2*$^{-/-}$ mice of different strain backgrounds. Although *rag2*$^{-/-}$ mice suffer a persistent *B. hermsii* infection, they survive for approximately 12 to 21 days, allowing us to assess the role of the innate immune system in control of *B. hermsii* bacteremia. For these experiments, we selected *rag2*$^{-/-}$ counterparts of the three strains that showed the greatest differences at day 3 postinfection when the infection was performed in wild-type mice. BALB/c *rag2*$^{-/-}$, C57BL/6 *rag2*$^{-/-}$, and C3H/HeN *rag2*$^{-/-}$ mice were infected i.v. with 10^5 *B. hermsii* strain DAH bacteria and bled every 12 or 24 h for 14 days. Bacteremia was monitored daily by dark-field microscopy of blood smears.

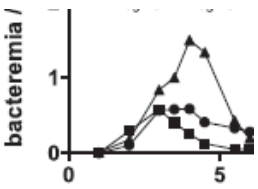

Figure 2-2. The efficiency of innate immune control of *B. hermsii* bacteremia varies with mouse strain. Two independent surveys (A and B) reveal a genetically defined course of *B. hermsii* spirochetemia in *rag2*$^{-/-}$ mice. Female mice between 4 and 6 weeks of age were infected i.v. in groups of five with 105 *B. hermsii* strain DAH bacteria. Five microliters of blood per day was collected from each mouse for 14 days postinfection for microscopic examination of blood smears. Inset shows spirochete numbers in bloodstreams of individual mice at day 4 postinfection, along with means and standard deviations for the groups. At this time point, C3H/HeN *rag2*$^{-/-}$ mice demonstrated significantly ($P < 0.001$, one-way ANOVA) higher levels of bacteremia than BALB/c *rag2*$^{-/-}$ or C57BL/6 *rag2*$^{-/-}$ mice.

The three strains examined exhibited significant differences in the level of bacteremia, and these differences are described below. Nevertheless, an overall triphasic

pattern of infection was apparent for each of the three inbred strains (Fig. 2-2). First, the mice suffered a single peak of bacteremia that was then partially controlled by day 6 postinfection. Second, this was followed by a period of four to six days in which the bacteremia was moderate and relatively consistent. Finally, bacteremia increased, and in two of the three strains, this increase was dramatic, leading to morbidity and mortality.

While the overall pattern of bacteremia showed some similarities among the three *rag2*$^{-/-}$ mouse strains, we were able to identify three periods over the course of the 14-day infection during which there were significant differences in bacteremia between the strains tested. First, at days 3.5 to 4 postinfection, we found that BALB/c *rag2*$^{-/-}$ mice suffered approximately 2.5-fold lower bacteremia than C3H/HeN *rag2*$^{-/-}$ mice, consistent with the relative susceptibilities of their wild-type counterparts, BALB/cByJ and C3H/HeJ mice, respectively. Interestingly, C57BL/6 *rag2*$^{-/-}$ mice were relatively resistant, suffering less than half the level of bacteremia ($P < 0.01$) seen in C3H/HeN *rag2*$^{-/-}$ mice (Fig. 2-2A, see inset), a finding in contrast to the results for wild-type C57BL/6 mice, which were relatively susceptible to *B. hermsii* infection at the first peak of bacteremia (Fig. 2-1). In addition, while none of the *rag2*$^{-/-}$ mice cleared *B. hermsii* infection, after the first peak of bacteremia, C57BL/6 *rag2*$^{-/-}$ mice were able to control bacteremia better and survived longer than either C3H/HeN *rag2*$^{-/-}$ or BALB/c *rag2*$^{-/-}$ mice.

Second, at approximately day 7 postinfection, BALB/c *rag2*$^{-/-}$ mice suffered an additional peak of bacteremia that was not observed with the other strains (Fig. 2-2A).

Thus, at this time point, BALB/c *rag2*$^{-/-}$ mice had 6-fold ($P < 0.001$) and 10-fold ($P < 0.001$) higher numbers of blood-borne bacteria than C3H/HeN *rag2*$^{-/-}$ and C57BL/6 *rag2*$^{-/-}$ mice, respectively. By day 9 postinfection, BALB/c *rag2*$^{-/-}$ mice were able to establish partial (albeit temporary) control of bacteremia. Finally, between days 12 and 14 post-infection, BALB/c *rag2*$^{-/-}$ mice displayed approximately 10-fold greater bacteremia than C57BL/6 *rag2*$^{-/-}$ mice ($P < 0.05$ at day 12 for all experiments). During this period of time, C3H/HeN *rag2*$^{-/-}$ mice had intermediate numbers of bacteria in the bloodstream. The increase in bacteremia in BALB/c *rag2*$^{-/-}$ and C3H/HeN *rag2*$^{-/-}$ mice corresponded to signs of clinical illness, such as listlessness and ruffled fur, prompting euthanasia of many of the mice (data not shown). Thus, although it has been established that an antibody response is critical for clearing *B. hermsii* infection in wild-type mice, the variation in response to infection by different strains of *rag2*$^{-/-}$ mice indicates that innate components of the immune system also contribute to the clearance of *B. hermsii* from the bloodstream.

B. hermsii induced thrombocytopenia and anemia.

Associated with the episodes of spirochetemia in mice are anemia and thrombocytopenia, which are also commonly observed in human patients (6, 62). Thus, in addition to monitoring bacteremia, in the second strain survey of *rag2*$^{-/-}$ strains (Fig. 2-2B), we also followed RBC and platelet counts by flow cytometry (see Materials and

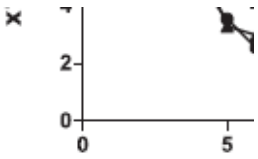

Figure 2-3. Thrombocytopenia and anemia in *rag2*$^{-/-}$ mice. Blood sampled from bacteremic *rag2*$^{-/-}$ mice was used for flow cytometry based measurement of platelets (A) and RBCs (B). Rapid initial reduction in the numbers of RBCs and platelets was followed by a recovery period in all 3 strains of mice examined. The minimum in the platelet numbers corresponded to the peak bacteremia at day 4. The minimum in the number of RBCs corresponded to the minimum bacterial loads following the initial peak of bacteremia.

Methods). Consistent with earlier analysis of *B. hermsii* infection in immunocompromised mice (6), all mice became thrombocytopenic. The kinetics of thrombocytopenia were similar across all mouse strains examined (Fig. 2-3A). Over the first three days, there was a rapid decrease in the number of platelets, with platelet counts reaching 10 to 20% of normal levels by day 3 postinfection, the time point of peak bacteremia. The platelet counts recovered very slightly over the next few days, but the mice remained severely thrombocytopenic. At day 12 postinfection, the average platelet count dropped even further. The platelet count profiles among all three strains of mice were highly similar, although the counts of BALB/c *rag2*$^{-/-}$ mice recovered slightly better

than those of the other strains ($P < 0.05$ for day 5). We conclude that the genetic background of these mice has little influence on the development and course of thrombocytopenia during relapsing fever infection.

Measurement of RBC counts indicated that infection affects the numbers of RBCs, but not in a fashion synchronous with changes in platelet numbers. We discerned three phases of anemia in immunocompromised mice. In the first phase, RBC counts in infected mice began to decline rapidly early in infection, although less precipitously than platelet counts (Fig. 2-3B). Unlike the platelet numbers, for which the period of rapid decline was limited to the first three days of infection, RBC numbers continued to decline until day 6. At this time point, all three strains of mice had only 30% to 45% of their normal number of RBCs. Interestingly, this time point corresponded to the point at which bacteremia was at a minimum following the initial peak of bacteremia at day 3 to 4 (Fig. 2-2). In the second phase, after day 6, the RBC count partially recovered and held steady for approximately four to six days, until about day 11 postinfection. Finally, the RBC count diminished in all three mouse strains through day 14, at which point the experiment was terminated due to frank illness of the animals. This third phase corresponded to an increase in bacteremia in all three strains. However, the degree of anemia did not directly correlate with the degree of bacteremia. For example, at day 12 postinfection, while both C3H/HeN *rag2*$^{-/-}$ and BALB/c *rag2*$^{-/-}$ mice had very high levels of bacteremia (>2 X 10^8) (Fig. 2-2), C3H/HeN *rag2*$^{-/-}$ mice had approximately 12-fold lower red blood cell counts than BALB/c *rag2*$^{-/-}$ mice ($P < 0.001$) (Fig. 2-3B). In addition, whereas the bacterial load in C57BL/6 *rag2*$^{-/-}$ mice at days 12 to 14 was

considerably lower than that in C3H/HeN *rag2*$^{-/-}$ and BALB/c *rag2*$^{-/-}$ mice, the C57BL/6 mice displayed a level of anemia intermediate between the levels in the other two strains. Therefore, our results indicate that changes in the number of RBCs following *B. hermsii* infection (Fig. 2-3B) do not simply reflect bacterial burdens and thus appear to be under independent genetic control.

Sex-specific differences in innate control of *B. hermsii* infection.

The gender of the host often influences the course and outcome of infection. To determine if innate immune control of *B. hermsii* infection has a sex-specific component, we compared the courses of infection in male and female littermates. We infected five male and five female littermates of the BALB/c *rag2*$^{-/-}$ and C57BL/6 *rag2*$^{-/-}$ strains with 10^5 *B. hermsii* strain DAH bacteria via tail vein injection. In both strains examined, we found that at the early time point (days 3 to 4), male littermates suffered 2- to 4-fold higher levels of bacteremia than their female counterparts (Fig. 2-4). In addition, peak bacteremia occurred 12 to 24 h later in male littermates than in females. At the middle and late time points, however, differences in bacteremia between male and female littermates were not significant. These data indicate a strong influence of gender on resistance to *B. hermsii* infection, particularly in the kinetics and effectiveness of early suppression of bacteremia.

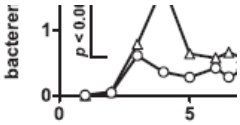

Figure 2-4. Male *rag2*$^{-/-}$ mice are more susceptible than females to *B. hermsii* infection in the early stages of infection. (A) BALB/c *rag2*$^{-/-}$ males suffered more severe bacteremia than females of the same strain at the early time point (day 4 postinfection). Peak bacteremia in males occurred 12 to 24 h later than that of female mice. (B) C57BL/6 *rag2*$^{-/-}$ males also suffered higher levels of bacteremia than female littermates at the early time point. However, bacteremia in C57BL/6 *rag2*$^{-/-}$ males was lower than that of BALB/c *rag2*$^{-/-}$ males at the same time point. Inset panels show peak bacteremia in the female cohorts only. All experiments, $n = 4$; comparisons by two-tailed *t* test.

Histopathology of infection.

To further characterize the course of *B. hermsii* infection in immunocompromised mice, we performed histopathological analysis of liver, spleen, and heart tissues from infected animals. The liver and spleen were selected on the basis of their role as primary sites of bacterial clearance. Additionally, in mice, liver and spleen are organs where ectopic hematopoiesis can occur. We also collected heart tissue, to determine if this organ is a site of spirochetal invasion, given that carditis is a prominent feature of *Borrelia burgdorferi* and relapsing fever *Borrelia* infections (32, 93).

Liver, spleen, and heart tissues were collected from animals infected with 10^5 CFU of DAH bacteria for various amounts of time. Hepatosplenomegaly has been

previously observed during *B. hermsii* infection of immunocompetent mice (6). Indeed, necropsies of *rag2*$^{-/-}$ animals selected for tissue harvest revealed dramatic hepato- and

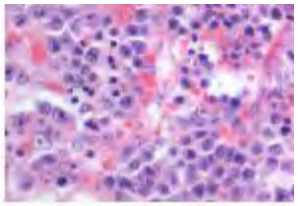

Figure 2-5. Histopathology of infected *rag2*$^{-/-}$ mice. Panels show H&E staining of liver (A) and spleen (B) sections of organs recovered from BALB/c *rag2*$^{-/-}$ animals infected with 10^5 CFU *B. hermsii* strain DAH bacteria. Numerous RBCs were found in tissue-resident macrophages (box), indicating extensive erythrophagocytosis. The presence of megakaryocytes in spleens of infected animals indicates ongoing extramedullary hematopoiesis.

splenomegaly at the late stages of infection in all strains examined. In addition, the spleens of BALB/c *rag2*$^{-/-}$ animals were more than two times larger than those of the C57BL/6 *rag2*$^{-/-}$ mice (data not shown).

Our histopathological analysis revealed that *B. hermsii* infection leads to the development of three processes that could contribute to splenomegaly. There were no

qualitative differences between spleens of *rag2*$^{-/-}$ mouse strains (data not shown), and to illustrate the results described below, we use representative images obtained using samples from BALB/c *rag2*$^{-/-}$ mice. First, analysis of H&E-stained sections revealed the expected influx of inflammatory cells in both livers and spleens of infected animals (Fig. 2-5). Second, spleens of infected animals had numerous megakaryocytes present (Fig. 2-5B, arrow), indicating extensive extramedullary hematopoiesis. Extramedullary hematopoiesis is often seen in chronic anemia and is consistent with the anemia of *B. hermsii* infection (Fig. 2-3A). Third and most striking, all sections examined demonstrated extensive erythrophagocytosis by resident tissue macrophages. Indeed, by day 6 postinfection, the majority of tissue macrophages contained large numbers of RBCs (Fig. 2-5A, box). Thus, one of the developments in the progression of *B. hermsii* infection in immunocompromised mice may be removal of RBCs by tissue-resident macrophages, leading to anemia. The extent of erythrophagocytosis in this nonquantitative assessment was similar in all inbred strains examined.

Localization of spirochetes by fluorescent *in situ* hybridization.

To determine the level of tissue invasion by spirochetes during infection and whether the erythrophagocytosis observed upon histological analysis might be reflected in specific associations between spirochetes and RBCs, we visualized spirochetes in tissues using FISH with a *Borrelia* genus-specific probe. FISH analysis revealed that by day 3 postinfection, spirochetes were found in all three of the tissues examined, i.e.,

heart, spleen, and liver. In the heart, spirochetes were visualized associated with blood vessel walls, as well as with cardiac muscle, oriented parallel to the muscle fiber (Fig. 2-6a, arrows). In the liver and spleen, RBCs were identified on the basis of their anuclear and highly autofluorescent morphology (see also stacked RBCs in the cardiac blood

Figure 2-6. FISH analysis of the time course of *B. hermsii* infection. *B. hermsii* spirochetes (orange [arrows and arrowheads]) could be detected in tissues (green background fluorescence) of infected BALB/c *rag2*$^{-/-}$ animals as early as 3 days after infection (a to c). By day 6, the spirochetes could be found associated with anuclear cells (d to f, arrowheads) and blood vessel walls (d and e, arrows). At the late stages of infection, numerous spirochetes were visible in cardiac muscle (g), liver (h), and spleen (i) tissue. DAPI nucleic acid stain (blue) reveals the host cell nuclei.

vessel, Fig. 2-6d), and many spirochetes were observed associated with these cells (Fig. 2-6a and b, arrowheads). By day 6, the numbers of spirochetes found in each tissue increased despite a reduction in numbers of blood-borne bacteria (Fig. 2-2). Spirochetes were readily seen associated with the blood vessel walls (Fig. 2-6d and e, arrows), and numerous spirochetes were observed wrapped around RBCs (Fig. 2-6d to f, arrowheads). By day 12 postinfection, *B. hermsii* numbers in the tissues increased further, correlating with the increased bacterial load in the blood and ultimately leading to severe infection and death of BALB/c *rag2*$^{-/-}$ and C3H *rag2*$^{-/-}$ animals. In the heart, spirochetes were observed within the heart muscle (Fig. 2-6g), and in the liver and, particularly, in the spleen, there were numerous spirochetes associated with RBCs (Fig. 2-6h and i). Thus, in addition to providing evidence that *B. hermsii* bacteremia is associated with significant invasion into tissues such as the heart, the results of our immunofluorescence examination indicate an association of spirochetes with RBCs, one that might underlie the extensive phagocytosis of red blood cells by tissue-resident macrophages.

Discussion

The genetic identity of the host plays a significant role in the control of a wide range of infectious diseases, from viral infections to colonization with parasites. The tick-borne spirochete *B. hermsii* is extremely well adapted to growth in the bloodstream of infected hosts and may therefore serve as a model in which to study the mechanisms of clearance of bacterial pathogens from the bloodstream. Although the analysis of *B. hermsii* infection has provided important insights into how bloodborne pathogens trigger a host immune response, particularly a robust antibody response, no studies of the contribution of the genetic component of the host to the course and outcome of *B. hermsii* infection have been performed. In the current study, by analyzing the course of *B. hermsii* infection in inbred strains of mice, we established that in fact, in the absence of a humoral response, the innate immune system is capable of partial control of *B. hermsii* infection. In addition, we found that the genetic background of the host contributes significantly to the severity of both bacteremia and anemia, prominent manifestations of infection.

To begin our analysis, we selected 5 common inbred commercially available mouse strains that have previously been demonstrated to display differential patterns of sensitivity to various pathogens (55). Our survey revealed significant strain specific differences in the progression of infection, in that bacteremia was 6-fold more severe in C57BL/6ByJ than in BALB/cByJ animals at day 3 after infection (Fig. 2-1). The high statistical significance ($P < 0.01$) of this finding suggests that inbred mouse strains are

sufficiently different in clearing *B. hermsii* from the bloodstream to allow for the identification of the genetic polymorphisms that likely account for this differential susceptibility.

It is well established that adaptive immunity, particularly a T-cell-independent antibody response, is critical for clearance of relapsing fever spirochetes. To determine if genetic differences in innate immunity contribute to the differences in susceptibility to infection that we observed in immunocompetent strains, we followed the course of *B. hermsii* infection in an analogous set of strains carrying a *rag2* deletion. *Rag2* knockout mice, like *scid* and *rag1*$^{-/-}$ animals, lack specific immunity, but their innate immunity is intact (139). We found that *rag2*$^{-/-}$ mice had higher levels of circulating spirochetes at the initial peak of bacteremia than their immunocompetent counterparts. This is likely due to a combined contribution of innate and adaptive immunity to the early response to *B. hermsii* infection in immunocompetent mice. Rapid response to *B. hermsii* early in the infection can be mediated by both marginal zone (MZ) B and B1 cells (2, 18). These T-cell-independent B-cell subsets can differentiate into plasmablasts within 48 h after exposure to bacteria or bacterial products and are therefore capable of generating a significant specific-antibody response, required for controlling bacterial burden, by 3 days postinfection (8, 98). Consistent with the results of our previous work with immunocompromised (*rag1*$^{-/-}$) mice, none of the animals fully cleared the infection, but each was capable of diminishing the bacterial load from an early peak, indicating that innate immune mechanisms were functioning to control infection (Fig. 2-2) (3). In addition, consistent with the relative susceptibility of immunocompetent C3H mice, C3H

rag2$^{-/-}$ mice did not control the initial peak of bacteremia as well as BALB/c *rag2*$^{-/-}$ mice, indicating that at least in part, the susceptibility of wild-type C3H *rag2*$^{-/-}$ mice reflects a relative defect in innate immunity. Interestingly, in direct contrast to the relative susceptibility of immunocompetent C57BL/6ByJ mice, female C57BL/6 *rag2*$^{-/-}$ mice were able to control the initial peak of bacteremia as well as BALB/c mice. Because these C57BL/6 mice are separated from their common progenitors by many generations, it is possible that the different patterns of response to *B. hermsii* infection of immunocompetent and immunocompromised mice are due to subline-specific genetic differences. In fact, we recently described C57BL/6 subline-specific differences in response to *L. monocytogenes* infection (61). Nevertheless, the detrimental effect of adaptive immune cells on innate immune function has been documented for the mouse model of *L. monocytogenes* infection (33). Therefore, it is possible that although the combined efforts of the innate and adaptive immune systems of wild-type C57BL/6 mice are capable of completely clearing *B. hermsii* infection, the innate immune response to *B. hermsii* by these mice is impaired at the early stages of infection.

The gender of the host can often influence susceptibility to infectious disease. In addition to the relevance of such sex specific phenotypes for understanding the pathogenesis of infection, for genetic studies it is important to know if the phenotype can be analyzed using entire cross populations or if the animals have to be segregated according to sex. To test the effect of gender, we compared the progression of *B. hermsii* infection in male and female C57BL/6 *rag2*$^{-/-}$ and BALB/c *rag2*$^{-/-}$ animals. For both strains, males had significantly higher and somewhat delayed initial peaks of bacteremia

compared to the initial peaks in female animals, indicating that the gender of the host plays a substantial role in control of *B. hermsii* bacteremia. While it would be difficult to test directly if similar gender-specific differences in susceptibility to relapsing fever exist in the human population, our observation provides a starting point for research in this direction.

In addition to bacteremia, relapsing fever patients commonly suffer from anemia and thrombocytopenia. The etiology of these changes in blood cell counts is not understood and could be multifactorial, but it is tempting to speculate that the proximity of these cells to high concentrations of bacteria in the blood promotes interactions that lead to host cell clearance. Indeed, episodes of thrombocytopenia correspond temporally with peaks of bacteremia, and the spirochetes bind to circulating platelets during infection (5). In addition, both *Borrelia crocidurae* and *B. hermsii* have been shown to bind to erythrocytes *in vitro* and in blood samples from infected animals (30, 68). Our examination of tissues provided data that support the hypothesis that the interaction of spirochetes with cellular components of blood influences the number of circulating cells. We found that liver-resident macrophages (Kupffer cells) contain significant numbers of phagocytosed and highly clustered RBCs (Fig. 2-5), and FISH analysis demonstrated that RBCs are frequently associated with spirochetes, some of which appear to wrap around the entire periphery of the cell (Fig. 2-6). These findings, along with the kinetics of anemia, are consistent with a speculative model in which RBC binding might contribute to the ability of animals to control bacteremia. According to this model, bacteremia is associated with spirochete-RBC interactions, leading to RBC clearance by

erythrophagocytosis, either due to RBC damage or to "bystander" clearance of RBCs stably bound to spirochetes. The phagocytosis of spirochetes and RBCs leads to hepatosplenomegaly, as well as to a decrease in bacteremia by day 6 of infection. This is followed by a partial recovery in RBC count as hematopoiesis (including extramedullary hematopoiesis) occurs, and a period of relatively consistent anemia and bacteremia. However, late in infection, e.g., by day 11 or 12, perhaps as the erythropoietic and/or phagocytic capacities of infected animals become exhausted, decreasing numbers of circulating RBCs and increasing numbers of blood-borne spirochetes are observed, leading to the terminal phase of infection. Thus, one might imagine that RBCs play a role in the initial control of *B. hermsii* infection by acting as a "sink" for circulating bacteria. Bergstrom and colleagues have suggested that RBC binding by *B. crocidurae* promoted a delay in the development of an adaptive immune response (30), and these models are not mutually exclusive.

Our results indicate that innate immunity plays a significant role in the control of *B. hermsii* replication and that this contribution is different in inbred mouse strains. In addition, our study lays the groundwork for a future thorough, unbiased genetic analysis of differential responses to *B. hermsii* infection in inbred mouse strains that will identify critical control elements in the regulation of innate immunity. Analysis of these elements will improve our understanding of innate immune response to blood-borne pathogens. In addition, since innate immunity contributes to defense against a wide range of pathogens, these findings are likely to contribute to our understanding of general mechanisms of host-pathogen interactions.

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CHAPTER III

Allelic variation of DbpA of the Lyme disease spirochete influences binding to decorin, dermatan sulfate, and cultured epithelial cells

Contributions

Yi-Pin Lin contributed to the experiments for figures 3-3B, 3-4 and 3-5. Joshua Fischer designed tables 3-2 and 3-3, and contributed to the design of tables 3-4 and 3-5.

Abstract

After transmission by an infected tick, the Lyme disease spirochete, *Borrelia burgdorferi* sensu lato, can colonize the mammalian skin and often disseminates systemically. The three major species of Lyme disease spirochete, *B. burgdorferi* sensu stricto, *B. garinii* and *B. afzelii*, are associated with different chronic manifestations of infection. Chronic colonization of multiple tissues is likely promoted by the ability to bind to components found in target tissues, and Lyme disease spirochetes utilize multiple adhesive molecules to interact with diverse mammalian components. The allelic variable surface lipoprotein decorin binding protein A (DbpA) promotes bacterial binding to the proteoglycan decorin and the glycosaminoglycan (GAG) dermatan sulfate. To assess allelic variation in the GAG-, decorin- and cell-binding activities of DbpA, we expressed different DbpA alleles in *B. burgdorferi* strain B314, a high-passage, noninfectious, that lacks *dbpA* and is not capable of binding mammalian cells. The alleles tested include representatives of *B. burgdorferi* sensu stricto, *B. garinii* and *B. afzelii*. Each of the DbpA alleles conferred upon *B. burgdorferi* strain B314 the ability to bind to cultured kidney epithelial (but not glial or endothelial) cells, as well as to purified decorin and dermatan sulfate. Nevertheless, allelic variation of DbpA was associated with dramatic differences in substrate binding activity. Whereas in most cases decorin and dermatan sulfate binding correlated well, one DbpA allele promoted differential binding to decorin and dermatan sufate, indicating that the two activities are not identical. Another allele with relatively low adhesive activity is derived from a strain that is known to cause disseminated

infection in mice, indicating that robust DbpA-mediated adhesive activity is not required for spread in the mammalian host.

Introduction

Lyme disease spirochetes of the genus *Borrelia* are transmitted to humans from a bite by an infected *Ixodes* tick. Transmission of spirochetes to humans from the infected tick can result in a local infection of the skin, typically giving rise to a characteristic rash termed erythema migrans. In the absence of antibiotic therapy, some strains may disseminate from the skin, via the blood , to multiple secondary sites including joints, heart, and brain, resulting in the varied clinical manifestations of Lyme disease such as arthritis, carditis and neuroborreliosis (for review, see (142)).

At least seven species of *Borrelia* are associated with Lyme disease (43, 63, 125, 129, 142), and are collectively referred to as *B. burgdorferi* (sensu lato). The three most common Lyme disease spirochetes are *B. burgdorferi* (sensu stricto), *B. garinii* and *B. afzelii*. *B. burgdorferi* (sensu stricto), herein referred to simply as *B. burgdorferi*, is the most prevalent Lyme disease spirochete in the United States. In Europe all three species are associated with Lyme disease *B. garinii* and *B. afzelii* predominate. The three species are each capable of causing long-term infection in humans, but are associated with different chronic manifestations: *B. burgdorferi* with Lyme arthritis, *B. garinii* with neuroborreliosis, and *B. afzelii* with the chronic skin lesion acrodermatitis (157). Additionally, within a species, there is apparently strain-to-strain variation in the ability to cause disseminated infection (155, 163). *B. burgdorferi* strains, typed on the basis of polymorphisms in the rRNA operon or the highly polymorphic *ospC* gene, have been shown to vary in their association with bloodstream infection in humans (53, 158, 162).

Although OspC, a surface lipoprotein, is required for experimental infection of the mouse (65), allelic variation of OspC cannot fully account for observed differences in the capacity to disseminate within the mammalian host by various *B. burgdorferi* strains (1). Thus, other virulence factors likely contribute to the apparent differences in tissue colonization and clinical manifestations exhibited by diverse Lyme disease spirochetes.

Attachment to host tissues is thought to be a critical step in the ability of many pathogens to disseminate within the mammalian host. *B. burgdorferi* attaches to a wide variety of cells *in vitro* including epithelial, endothelial and glial cells, platelets and lymphocytes (45, 49, 59, 60, 147, 148). In addition, multiple host cell and extracellular matrix molecules are recognized by *B. burgdorferi* such as integrins (39), fibronectin (24, 64, 118), laminin (25, 152), collagen (171), and proteoglycans (67, 78, 86). Proteoglycans consist of a protein core covalently linked to one or more glycosaminoglycan (GAG) chains, which are long linear repeating disaccharides (for review, see (94)). GAGs are typically quite heterogeneous in size and vary structurally between tissues and host species (116). Different GAGs can be segregated into classes based on epimerization of the glycan chain, location and degree of sulfation, and sensitivity to enzymatic cleavage by different lyases.

We previously showed that diverse spirochetes recognize distinct classes of GAGs, and differences in GAG-binding specificity can lead to differences in the types of mammalian cells recognized (113). For example, *B. burgdorferi* N40 strain, which recognizes heparan sulfate and dermatan sulfate GAGs, binds efficiently to glial and

endothelial cells *in vitro*, while *B. afzelii* strain VS461, which predominately recognizes dermatan sulfate, binds selectively to glial cells (113). These findings suggest that strain specific differences in GAG recognition by Lyme disease spirochetes might influence the specificity of host cell attachment and thus tissue colonization during infection.

B. burgdorferi encodes multiple GAG binding proteins, including the highly related decorin binding proteins (Dbp) A and B, which were first identified on the basis of their ability to bind decorin, a chondroitin/dermatan sulfate proteoglycan that "decorates" collagen fibers in mammalian tissues (66, 67, 70). These proteins, when expressed in a noninfectious and otherwise nonadherent *B. burgdorferi* strain, were shown to be sufficient to promote spirochetal attachment to isolated dermatan sulfate GAGs as well (57). The *dbpA* (and presumably *dbpB*) gene is not expressed in the unfed tick, but transcription is induced upon tick feeding (75). In addition, DbpA and B are required for maximal infectivity and dissemination in both immunodeficient and immunocompetent mice (21, 138, 160). These findings are consistent with the hypothesis that spirochetal binding to extracellular matrix mediated by DbpA and DbpB promotes colonization of the mammalian host.

DbpA and DbpB are related proteins that each bind to dermatan sulfate, but differ in their GAG and mammalian cell type binding specificities (57). In addition, while DbpB is highly conserved among Lyme disease spirochete strains, DbpA is highly polymorphic (123), and recombinant derivatives of DbpA allelic variants differ in decorin binding (115). In this study, we found that allelic variation of DbpA is associated with

dramatic differences in the ability of this adhesin to promote bacterial attachment to GAGs and mammalian cells.

Experimental procedures

Bacterial strains and cells lines. The high-passage *B. burgdorferi* strain B314 (128) was the generous gift of Tom Schwan (Rocky Mountain Labs, Hamilton, MT). *B. burgdorferi* strains N40 clone D10/E9 ("$N40_{D10/E9}$"), B356, B31, and 297, *B. garinii* strain PBr, and *B. afzelii* strain VS461 have been described previously (39, 58). B314 and its derivatives were cultured at 33°C in BSKII complete medium (10), supplemented with 200 μg/ml kanamycin where appropriate. For a list of bacterial strains used in this study, see Table 3-1. 293 (human kidney epithelial) cells, C6 (rat glioma), and EA-hy926 (human umbilical vein endothelial) cells were cultured as described previously (113).

Table 3-1. Bacterial strains used /generated in this study.

Strain	Species	Origin	Reference
N40 (clone D10/E9)	*B. burgdorferi*	Tick, United States	Coburn *et al.* (9)
B356	*B. burgdorferi*	Skin	Wang *et al.* (51)
B31	*B. burgdorferi*	Tick, Switzerland	Barbour *et al.* (3) Fraser et al. (16)
297	*B. burgdorferi*	Human, CSF, United States	Leong *et al.* (28)
PBr	*B. garinii*	Human, CSF, Germany	Coburn *et al.* (28)
VS461	*B. afzelii*	Tick, Switzerland	Marconi *et al.* (31)
B314/pJF21	*B. burgdorferi*	This study	
B314/pN40	*B. burgdorferi*	This study	
B314/pB356	*B. burgdorferi*	This study	
B314/pB31	*B. burgdorferi*	This study	
B314/p297	*B. burgdorferi*	This study	
B314/pPbr	*B. burgdorferi*	This study	
B314pVS461	*B. burgdorferi*	This study	

Plasmids and cloning. For initial sequence analysis, *dbpA* from *B. burgdorferi* strains N40, B356, B31 and 297, from *B. afzelii* strain VS461 and from *B. garinii* strain PBr were amplified by PCR from genomic DNA using primers and reaction conditions described previously (123). Briefly, the forward primer, 10F4 5'-GTGGTTAAGGAAAAACAAAA-3', is homologous to a sequence in the highly conserved *dbpB* gene (123), and the reverse primer, 5R1 5'-CCAAATAACATCAAAAAGGA-3', is homologous to a sequence downstream of *dbpA*. PCR with these primers resulted in the generation of amplicons approximately 1kb in size, which were inserted into pCR-XL-TOPO vector (Invitrogen) and then sequenced. Nucleotide sequence analysis showed that *dbpA* cloned from strains B31, 297, PBr and VS461 were identical to previously published sequences (123, 164). DbpA from strain $N40_{D10/E9}$ was found to be 95% identical to DbpA from an independent clone of N40 (123) and is for clarity referred to as $DbpA_{N40\text{-}D10/E9}$ in this study.

For expression in strain B314, each *dbpA* allele was amplified from genomic DNA by PCR using primers listed in Table 1 from the transcription initiation codon to its cognate termination codon. These amplicons were then cloned into a modified shuttle vector, pJF21, (57) using engineered SalI and BamHI sites at the 5' and 3' ends respectively, and sequenced with M13F and M13R primers. For production of recombinant proteins, *dbpA* from strains B31, $N40_{D10/E9}$, PBr, and VS461, minus their lipoprotein signal sequences, were amplified by PCR using primers described in Table 3-2 and cloned into pET15b (Novagen, Madison, WI).

Table 3-2. Primers used in this study. Underlined sequences denote restriction sites.

Purpose	Name	Primer Sequence*	Nucleotide position
B314/pDbpA$_{N40}$	AN40F	5'-ACGCGTCGACATGAATAAATATCAAAAAACTTTC-3'	1-24
	AN40R	5'-CGCGGATCCTTAGTTATTTTTGCATTTTTCATCAGT-3'	559-582
B314/pDbpA$_{B356}$	A356F	5'-ACGCGTCGACATGAATAAATATCAAAAAACTTTC-3'	1-24
	A356R	5'-CGCGGATCCTTAGTTATTTTTGCATTTTTCATC-3'	562-585
B314/pDbpA$_{B31}$	AB31F	5'-CGGTCGAC ATGATTAAATGTAAT-3'	1-15
	AB31R	5'-CGGGATCC TTAGTTATTTTTGCA-3'	562-576
B314/pDbpA$_{297}$	A297F	5'-ACGCGTCGACATGATTAAATGTAATAATAAAACT-3'	1-24
	A97R	5'-CGCGGATCCTTACGATTTAGCAGTGCTGTCTTC-3'	541-564
B314/pDbpA$_{PBr}$	APBrF	5'-ACGCGTCGACATGATTAAATATAATAAAATATTG-3'	1-24
	APBrR	5'-CGCGGATCCTTATGTAGTAGTAGCAGTTTTGGC-3'	535-558
B314/pDbpA$_{VS461}$	AVS461F	5'-ACGCGTCGACATGATTAAATATAATAAAATTATA-3'	1-24
	AVS461R	5'-CGGGATCCTTATTTTTGATTTTTAGTTTGTTTTTCTTTAATGTTTTCC-3'	487-510
BL21/pHis-DbpA$_{B31}$	B31HisDbpAF	5'-GCGGATCCGGACTAACAGGAGCAACA-3'	76-93
	B31HisDbpAR	5'-CGCTCGAGTTAGTTATTTTTGCATTT-3'	559-576
BL21/pHis-DbpA$_{PBr}$	PBrHisDbpAF	5'-GCGGATCCGGCTTAACAGGAGAAACT-3'	64-81
	PBrHisDbpAR	5'-CGCTCGAGTTATGTAGTAGTAGCAGT-3'	541-558
BL21/pHis-DbpA$_{VS461}$	5VS461DbpA	5'-GGAATTCCATATGAGTTTAACAGGAAAAGCTAGATTGGAA-3'	64-90
	AVS461R	5'-CGCGGATCCTTATTTTTGATTTTTAGTTTGTTTTTCTTTAATGTTTTCC-3'	487-510

Purification of human decorin. Recombinant human decorin, a generous gift from David Mann (MedImmune, Inc.), was purified from stably transfected Chinese hamster ovary cells as described (77).

Generation of recombinant proteins and antisera. Plasmids encoding recombinant His-tagged DbpA proteins were transformed into *E. coli* BL21, and protein expression was induced with 1mM IPTG. Bacteria were lysed and proteins were purified by nickel affinity chromatography as described in the manufacturer's instructions (Novagen). Five week old Balb/C mice were immunized with 100 μg of His-DbpA$_{B31}$, His-DbpA$_{N40\text{-}D10/E9}$,

His-DbpA$_{PBr}$, or His-DbpA$_{VS461}$ in complete Freund's adjuvant. The animals were boosted twice with 100 μg of the same proteins in incomplete Freund's adjuvant at two week intervals and antisera were prepared after terminal cardiac puncture.

Attachment of immobilized recombinant DbpA proteins to biotinylated glycosaminoglycans. Dermatan sulfate (Calbiochem), heparin and chondroitin-6-sulfate (Sigma) were biotinylated using EZ-Link Biocytin Hydrazide (Pierce) and then dialyzed in PBS using a Slide-A-Lyzer cassette (Pierce) with 10,000 kD MW cutoff. 96-well microtiter plates (Linbro) were coated overnight at 4°C with 1 μg of his-tagged recombinant DbpA in PBS. The next day, proteins were removed and wells were washed twice with PBS and then blocked with 1% BSA in PBS for 1 hour at room temperature. Blocking buffer was removed and 50 μl of each biotinylated GAGs, diluted to a final concentration of 100 μg/ml 1% BSA in PBS, was added to wells and incubated for 2 hours. After washing, bound GAGs were detected by ELISA using horseradish peroxidase tagged anti-biotin antibody.

Transformation of *B. burgdorferi* B314. Electrocompetent B314 spirochetes were prepared and transformed as described (103). Briefly, 100 ml of mid-log phase cultured spirochetes were harvested and washed twice in electroporation solution (EPS, 15% v/v glycerol, 0.27M sucrose) and resuspended in 100μl of EPS. 30-40 μg of plasmid DNA was added to the suspension and electroporated at 2200 volts in a 0.2cm cuvette, and then cultured in BSKII complete medium at 33°C for 24 hours. The transformation mix was added to 1.7% analytical grade agarose (Invitrogen) and plated onto a 1.5X

BSKII/agarose bottom layer in a sterile 100 X 20 tissue culture dish (Corning) in the presence of kanamycin (200 μg/ml), and plates were incubated at 34°C in a 2% CO_2 atmosphere for 2 weeks. Colonies were picked and cultured at 33°C to mid-log phase density in BSKII complete medium containing kanamycin (200 μg/ml).

Proteinase K treatment, SDS-PAGE and Western blotting. To detect DbpA proteins, lysates from 5 X 10^7 spirochetes were separated by 15% SDS-PAGE. DbpA and FlaB were identified by immunoblotting using polyclonal antibodies against the appropriate DbpA (diluted 1:5000) or a monoclonal antibody, CB1 (a gift from J. Benach, Stony Brook University, Stony Brook, NY) against FlaB (diluted 1:500), respectively. Surface localization of DbpA proteins in recombinant B314 strains was determined as previously described (118). Briefly, 5 X 10^7 spirochetes were centrifuged and pellets were washed twice in PBS + 2% BSA. After the final wash, pellets were gently lifted with 5mM $MgCl_2$ in PBS supplemented with 4 mg/ml Proteinase K (Sigma) or buffer only and incubated at room temperature for 30 minutes. To inactivate Proteinase K, 150 μg of phenylmethylsuphonyl fluoride (PMSF) was added to each pellet. Pellets were washed twice with PBS + 0.2% BSA, lysed and separated by 15% SDS-PAGE. DbpA proteins were identified by immunoblotting as described above.

Triton X-114 fractionation. Membrane fractions of B314 expressing DbpA were prepared as described previously (23). Briefly, *Borrelia* cultures were grown to log phase as determined by counting spirochetes by darkfield microscopy and 10^9 spirochetes were harvested and washed twice with 0.2% BSA in PBS. Spirochetes were then

resuspended in 2% Triton X-114 and allowed to incubate overnight with rocking at 4°C. The following day, insoluble material was removed by centrifugation and supernatants were phase separated as follows: Supernatants were warmed to 37°C for 15 minutes, and then centrifuged at 13,000 rpm for 15 minutes to separate the aqueous and detergent fractions. The aqueous phase was discarded and the detergent phase was washed three times by adding cold PBS to original volume and rewarming and recentrifuging as above in the phase separation step. After the final wash, proteins were precipitated by adding 9 volumes of cold 100% ethanol, incubating overnight at -20°C, and recovered by centrifugation. After washing with 90% ethanol, proteins were resuspended in PBS. Protein concentration was determined by BCA assay (Pierce). 15 μg of total protein from the outer membrane (detergent) fraction was then separated on a 15% SDS-PAGE gel and stained with Coomassie blue.

Attachment of spirochetes to mammalian cells. Radiolabled bacteria were prepared by growing spirochetes at 33°C in BSKII complete medium supplemented with 60 μCi/ml of (^{35}S)-methionine. When cultures achieved mid-log phase (approximately 5 X 10^7/ml), bacteria were harvested at 10,000 X g and pellets were washed twice with 0.2% BSA in PBS. Labeled spirochetes were then stored as aliquots at -80°C in BSK-H (Sigma) containing 20% glycerol.

One day before each assay, mammalian cells were lifted with 0.5% trypsin, 0.5mM EDTA (Invitrogen) and plated in 96-well break-apart microtiter plates (Nunc) which were previously UV-sterilized and coated with MBP-Inv497, a maltose binding

protein fusion containing the cell binding domain of the invasion protein from *Y. pseudotuberculosis* (85). Frozen aliquots of radiolabeled *B. burgdorferi* were thawed and resuspended at 1 X 10^8 cells/ml in BSK-H and incubated at room temperature for 2 hours. Prior to addition of radiolabeled spirochetes, cell monolayers were washed twice with PBS. Radiolabled spirochetes were then diluted 1:3 into GHS buffer (10mM glucose, 10mM hepes, 50mM NaCl, pH 7.0) and added in quadruplicate wells at 1 X 10^6 spirochetes/well. To enhance spirochete-cell contact, plates were centrifuged at 1000 rpm for 5 minutes and then rocked at room temperature for 1 hour. Unbound spirochetes were removed by washing wells four times with 0.2% BSA in PBS. Plates were then air-dried and the percentage of bound bacteria in each well was determined by liquid scintillation. Each strain was tested for cell binding in three to five independent experiments.

Enzymatic removal of specific classes of GAGs. Monolayers were incubated for 2 hours with 0.5U/ml heparinase I, heparitinase, or chondroitinase ABC (Sigma) at 37°C in RPMI supplemented with 1% BSA, 10^{-2} TIU/ml aprotinin and 165 μg/ml PMSF. After washing the monolayers with PBS, radiolabeled spirochetes were added to pretreated monolayers as described above.

Inhibition of binding with exogenous GAGs. Radiolabeled spirochetes were prepared as described above and were incubated for 30 minutes at room temperature in BSK-H supplemented with between 80 ng/ml and 6.25 mg/ml GAGs. Following incubation, spirochetes were diluted 1:3 in GHS buffer before addition to cell monolayers.

Attachment of radiolabeled bacteria to purified GAGs and decorin. Prior to each assay, wells from Nunc 96-well break-apart microtiter plates were coated with either a titration of purified human decorin (1.25-0.156 μg/ml) or dermatan sulfate (2.5-0.625 mg/ml) in PBS at 4°C overnight. Wells were washed 3 times with 0.05% Tween-20 in PBS. Wells were then blocked with 1% BSA in PBS for 1 hour at room temperature. After removal of the blocking buffer, radiolabeled *B. burgdorferi* were added to the wells as described above.

Results

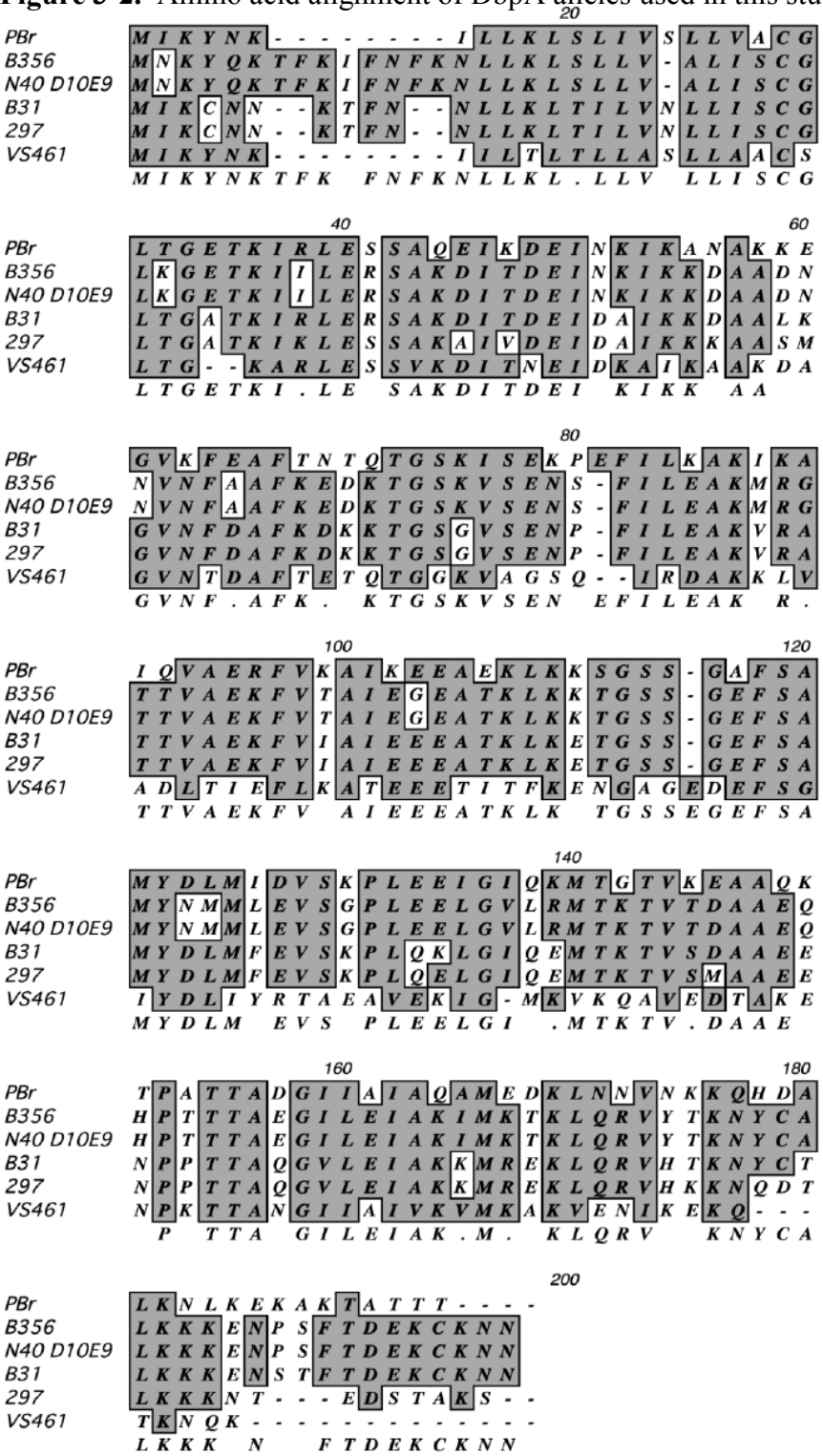
Ectopic expression of diverse DbpA alleles on the surface of a non-adherent *B. burgdorferi* strain.

We previously showed that recombinant DbpA from *B. burgdorferi* strain N40 clone D10/E9 ($N40_{D10/E9}$) bound to purified GAGs (57, 84). To determine if GAG binding activity is a common property of diverse DbpA alleles, here we examined DbpA from a variety of Lyme disease spirochetes. *B. burgdorferi* strain B31 is the type strain for this organism (58), *B. garinii* strain PBr displays high levels of the hemagglutination activity that is associated with GAG binding (86), and strain VS461 is a representative of *B. afzelii* (97). DbpA from strain $N40_{D10/E9}$ was found to be 95% identical to DbpA from an independent clone of N40 (123) and for clarity is referred to here as $DbpA_{N40\text{-}D10/E9}$. These DbpA alleles share as little as 62% identify (Table 3-3; Fig. 3-2).

	B.b.				*B.g.*	*B.a.*
	N40	B356	B31	297	PBr	VS461
N40	100	99	78	75	70	62
B356		100	78	72	67	56
B31			100	90	69	58
297				100	71	59
PBr					100	63
VS461						100

Table 3-3. DbpA alleles used in this study. A pairwise comparison of amino acid identities between DbpA alleles is shown. *B.b. = Borrelia burgdorferi*, *B.g. = Borrelia garinii, B.a. = Borrelia afzelii*. Dotted lines indicate groups of alleles which share significant homology.

Figure 3-2. Amino acid alignment of DbpA alleles used in this study.



Immobilized recombinant DbpA alleles were tested for their ability to bind to biotinylated GAGs. DbpA from each strain bound to heparin as well as to dermatan sulfate, but not to a control GAG, chondroitin-6-sulfate (Fig. 3-1).

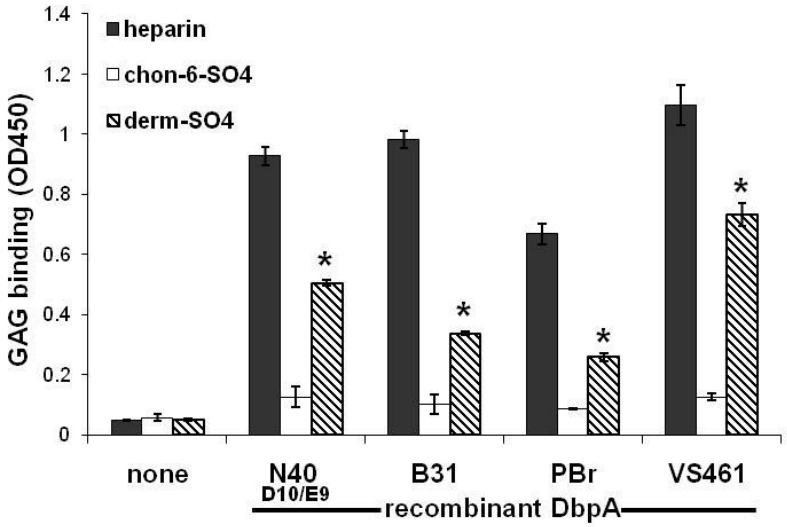


Figure 3-1. Recombinant DbpA proteins bind to biotinylated GAGs.

Microtiter wells were mock-coated (with PBS) or coated with His-tagged $DbpA_{N40}$, $DbpA_{B31}$, $DbpA_{PBr}$, and $DbpA_{VS461}$ proteins were probed with biotinylated heparin, dermatan-sufate or chondroitin-6-sulfate (see Materials and Methods). After washing, bound GAGs were detected by ELISA using α-biotin antibody conjugated to horseradish peroxidase and colorimetric substrate (TMB). Binding is reflected by absorbance at 450 nm. Each bar represents the mean of four independent determinations ± SD. [Asterisks indicate binding was significantly ($p < 0.05$, student's t test) higher than binding to mock-coated wells.]

Some differences in the apparent efficiency of GAG binding were noted among the different DbpA alleles, suggestive of allele-specific differences in GAG binding activity.

Given that it is not clear how faithfully the ability of recombinant DbpA to bind GAGs reflects the activity of DbpA when expressed on the spirochetal surface, we next tested the adhesive function of DbpA alleles on the surface of *B. burgdorferi*, taking

advantage of the fact that ectopic expression of $DbpA_{N40}$ is sufficient to confer spirochetal adhesiveness (128). *B. burgdorferi* strain B314 is a high passage strain that lacks all discernible linear plasmids, including lp54, which encodes the *dbpBA* operon, and appears to be incapable of binding to GAGs or cultured mammalian cells. Alleles of *dbpA* were cloned into the expression shuttle vector pJF21 3' to the *ospC* promoter (57, 144), which is highly active in strain B314 and is predicted to facilitate high-level transcription (128). Recombinant plasmids were then transformed into *B. burgdorferi* strain B314. In addition to the alleles of *B. garinii* PBr, *B. afzelii* VS461, and *B. burgdorferi* $N40_{D10/E9}$ and B31, we also analyzed *dbpA* from *B. burgdorferi* 297, a commonly studied strain, and from *B. burgdorferi* B356, a skin biopsy isolate that is incapable of causing persistent disseminated infection in mice(155) To confirm that these DbpA alleles were expressed in strain B314, bacterial outer membrane proteins were isolated by Triton X114 extraction and immunoblotted using mouse anti-DbpA antisera. As expected, each recombinant strain harboring a recombinant plasmid encoding *dbpA* was found to produce DbpA, whereas B314 harboring the pJF21 vector control did not (Fig. 3-3A). Proteinase K digestion of intact spirochetes resulted in complete or near-complete loss of reactivity to DbpA antisera in immunoblot analysis, indicating that all DbpA alleles were exported to the surface of strain B314. As predicted, the periplasmic protein FlaB remained intact, indicating the integrity of the outer membrane during Proteinase K digestion (Fig. 3-3B).

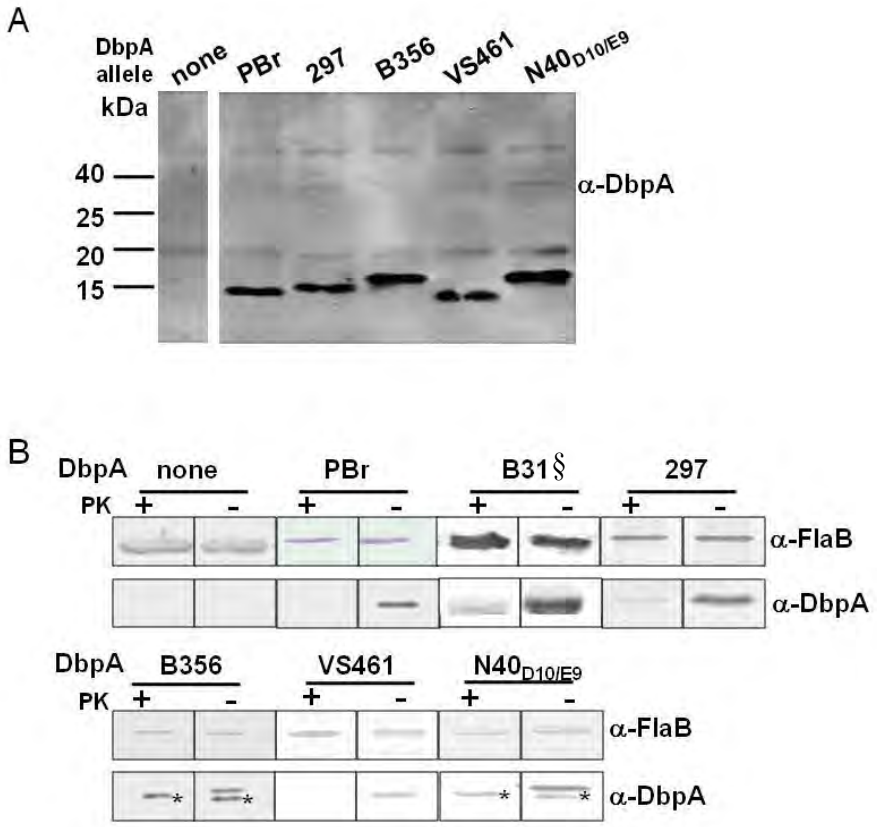


Figure 3-3. DbpA alleles from diverse Lyme disease spirochetes are expressed on the surface of a non-adherent *B. burgdorferi* strain.

A) Triton X-114 extractions of *B. burgdorferi* strain B314 expressing the indicated DbpA allele were subjected to 15% SDS-PAGE and immunoblotted with a mixture of antisera raised against $DbpA_{B31}$, $DbpA_{N40}$, $DbpA_{PBr}$, and $DbpA_{VS461}$.

B) Spirochetes expressing DbpA protein were digested with Proteinase K (pK) or incubated in PBS (Mock). Lysates from 5 X 10^7 treated bacteria were separated by 15% SDS-PAGE and DbpA and FlaB proteins were identified by Western blot. The detection of a Proteinase K-resistant band in B314/$pDbpA_{B356}$ and B314/$pDbpA_{N40}$ is a consistent finding and may represent intracellular DbpA protein that has not been lipidated. Flagellin, a periplasmic protein, was blotted for as a control. §This strain was analyzed in a separate experiment.

DbpA displays allelic variation in the recognition of dermatan sulfate on the surface of cultured epithelial cells.

$DbpA_{N40\text{-}D10/E9}$ was previously shown to promote attachment to cultured epithelial cells but not to endothelial nor glial cells (57). Therefore, recombinant B314 strains

expressing different DbpA alleles were tested for gain-of-function in attachment to 293 epithelial, EA-Hy926 endothelial, and C6 glial cells. Similar to our previous analysis of $DbpA_{N40\text{-}D10/E9}$, all DbpA alleles promoted spirochete attachment to cultured epithelial cells but not glial (Fig. 3-4) or endothelial cells (data not shown).

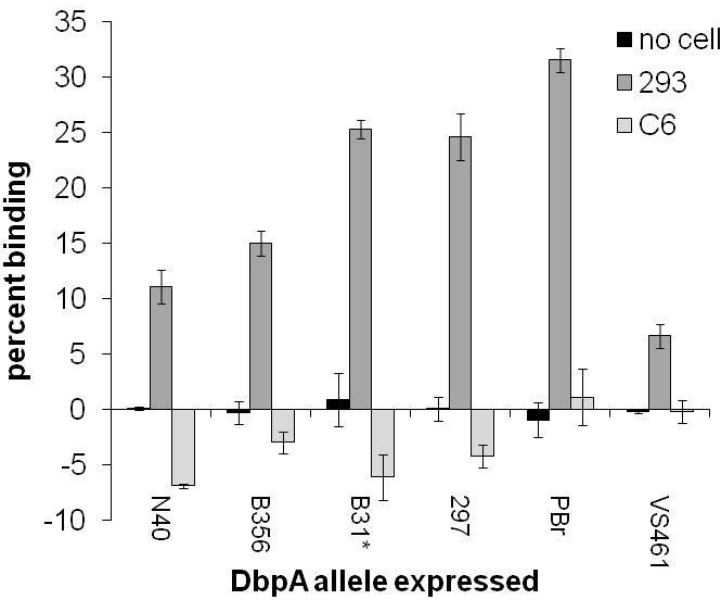


Figure 3-4. DbpA-mediated binding to cultured mammalian cells is variable among alleles and is cell type specific. Radiolabeled B.burgdorferi strain B314 spirochetes expressing the indicated DbpA allele were added to wells containing 293 epithelial cells, C6 glioma cells or no cells. Each bar represents the mean (± standard deviation) of four independent determinations with background binding (B314 expressing no DbpA) subtracted. Asterisk indicates that this strain was analyzed in a separate experiment.

$DbpA_{PBr}$ promoted the highest levels of attachment to epithelial cells. $DbpA_{297}$ and $DbpA_{B31}$ mediated spirochetal binding less efficiently than $DbpA_{PBr}$, but more efficiently than $DbpA_{N40\text{-}D10/E9}$, $DbpA_{B356}$ and $DbpA_{VS461}$. Complementation of the non-adherent strain B314 gave reproducible results across multiple assays, and independently derived clones expressing the same allele, either $DbpA_{N40\text{-}D10/E9}$ or $DbpA_{VS461}$, displayed similar binding to 293 epithelial cells, purified decorin and dermatan sulfate (Fig. 3-5). These results suggested that the binding phenotype measured in this way reflected the allele rather than the particular clone analyzed.

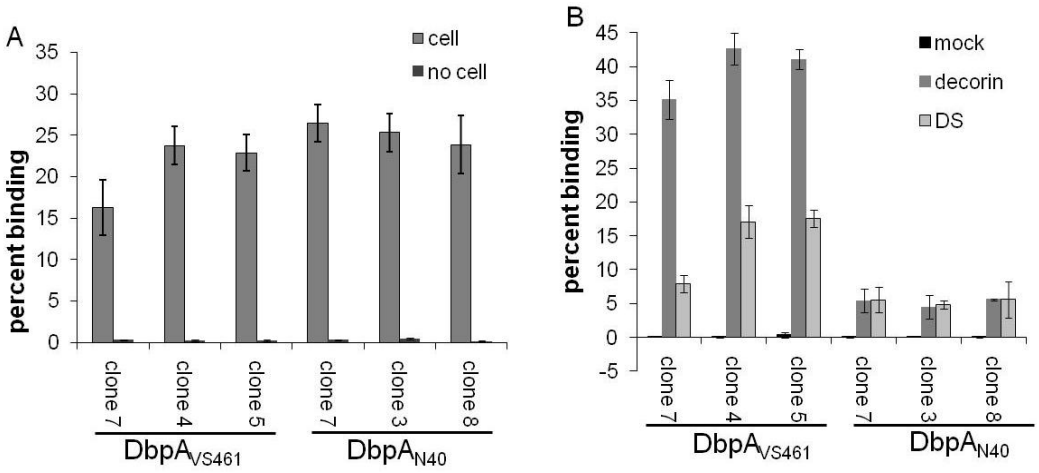


Figure 3-5. Independently derived clones expressing the same allele of DbpA display similar in vitro binding phenotypes. Radiolabeled *B.burgdorferi* strain B314 spirochetes expressing the indicated DbpA allele were added to wells containing A) 293 epithelial cells, or no cells; or B) decorin, dermatan sulfate or PBS (mock) coated wells. Each bar represents the mean (± standard deviation) of four independent determinations.

To determine if GAGs promoted binding of the recombinant B314 strains to 293 cells, monolayers were digested with specific lyases to remove different classes of GAGs from the cell surface. We previously showed that spirochetal binding to 293 cells mediated by DbpA$_{N40\text{-}D10/E9}$ required dermatan sulfate (57). Consistent with this, removal of dermatan/chondroitin sulfates with chondroitinase ABC virtually eliminated attachment promoted by all DbpA alleles, where as removal of heparin/heparan sulfates with heparinase or heparitinase had no significant effect (Table 3-4).

Plasmid	% Binding[b]			
	Mock digestion[a]	Hep.	Hpt.	Chon. ABC
Vector	2.9 ± 0.38	NA	NA	NA
$pDbpA_{PBr}$	42.3 ± 2.5	38.4 ± 6.0	42.6 ± 1.1	1.1 ± 2.0*
$pDbpA_{B356}$	17.0 ± 4.8	17.6 ± 0.9	23.6 ± 5.5	0.2 ± 0.2*
$pDbpA_{297}$	33.7 ± 6.6	34.8 ± 6.6	42.3 ± 4.5	2.0 ± 1.2*
$pDbpA_{N40\text{-}D10/E9}$	10.3 ± 1.4	8.3 ± 2.5	10.2 ± 1.7	0.6 ± 0.4*
$pDbpA_{VS461}$	6.6 ± 0.7	4.6 ± 1.6	5.5 ± 0.5	0.5 ± 0.3*

[a]Binding of radiolabeled transformants to confluent monolayers was determined as described previously. Each point represents the mean of four independent determinations ± SD. For all strains, less than 2% of bacteria bound to identically treated wells without mammalian cells (not shown).

[b]Binding to wells was determined after pre-treatment ofepithelial cells with the indicated lyase. Hep., Heparinase; Hpt., Hepartinase; Chon. ABC, Chondroitinase ABC; NA, Not Applicable. For all strains tested, binding was inhibited only by Chondroitinase ABC digestion and significant ($P<0.05$) differences in binding to mock-versus lyase-treated monolayers were determined by *t*-test analysis and are indicated by asterisks.

Table 3-4. GAG-binding by DbpA alleles from diverse Lyme disease can be inhibited by cleavage of chondroitin sulfate GAGs from the surface of epithelial cells.

In addition, exogenous dermatan sulfate, but not heparin or chondroitin-6-sulfate, significantly impaired cell binding by recombinant B314 strains expressing DbpA from strains B356, 297 or PBr (Table 3-5), suggesting that cell attachment was associated with GAG binding activity.

Plasmid	% Binding[b]			
	No inhibitor[a]	Hep	C6S	DS
Vector	2.9 ± 0.9	NA	NA	NA
$pDbpA_{PBr}$	57.5 ± 8.3	59.1 ± 3.8	58.8 ± 7.4	32.9 ± 8.5*
$pDbpA_{297}$	42.5 ± 5.6	33.0 ± 4.4	47.6 ± 6.9	26.9 ± 3.0*
$pDbpA_{B356}$	15.6 ± 3.8	22.6 ± 1.4	18.4 ± 4.2	3.8 ± 0.5*

[a]Binding of radiolabeled transformants to confluent monolayers was determined as described previously. Each point represents the mean of four independent determinations ± SD. For all strains, less than 2% of bacteria bound to identically treated wells without mammalian cells (not shown).

[b]Binding to wells was determined after pre-treatment of bacteria with the indicated soluble GAG DS, dermatan sulfate; Hep, Heparin; C6S, Chondroitin-6-sulfate; NA, Not Applicable. For all strains tested, binding was inhibited only by dermatan sulfate pre-treatment and significant ($P<0.05$) differences in binding to mock versus GAG-treated bacteria were determined by *t*-test analysis and are indicated by asterisks.

Table 3-5. DbpA alleles from diverse Lyme disease spirochetes specifically recognize dermatan sulfate on epithelial cells.

DbpA displays allelic variation in the ability to promote spirochetal attachment to purified decorin or dermatan sulfate.

We found previously that $DbpA_{N40}$ was able to confer binding to purified dermatan sulfate to strain B314 (57). To determine whether the variation in the ability of different DbpA alleles is associated with differences in their ability to promote binding to purified GAGs, we tested for binding of the recombinant B314 strains to microtiter wells coated with increasing concentrations of dermatan sulfate. Of all the DbpA-expressing strains, B314 expressing $DbpA_{PBr}$ bound most efficiently to dermatan sulfate at all concentrations tested (Fig. 3-6, right panel). $DbpA_{297}$ and $DbpA_{B31}$ promoted binding somewhat less efficiently than $DbpA_{PBr}$, but more efficiently than $DbpA_{VS461}$, $DbpA_{B356}$, or $DbpA_{N40\text{-}D10/E9}$ (Fig. 3-6 right panel). Binding mediated by the latter three alleles, while not robust, was nevertheless well above the background levels observed for strain B314 harboring control vector. Thus, variation in the ability of the DbpA alleles to promote binding to purified dermatan sulfate correlated with their ability to promote binding to 293 cells (compare Figs. 3-4 and 3-6 right panel), suggesting that 293 cell

attachment reflects dermatan sulfate binding activity.

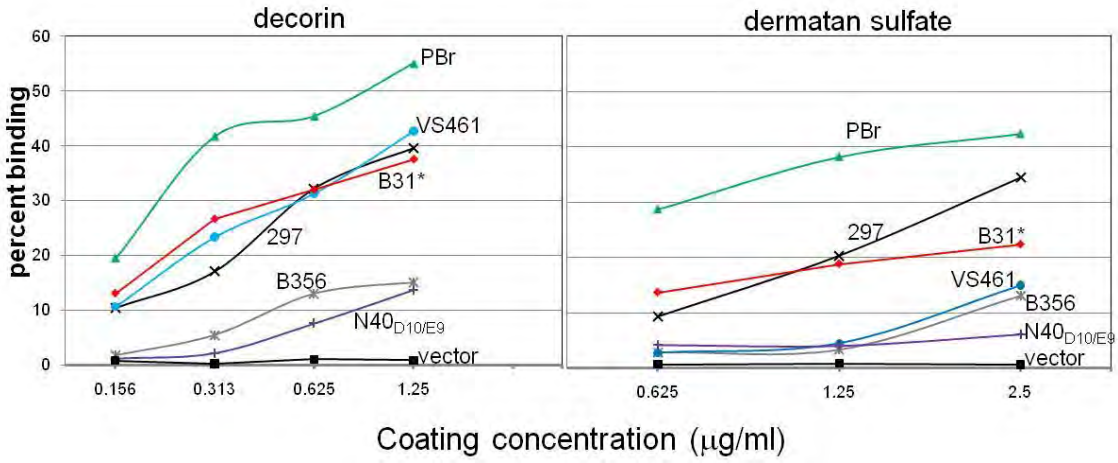


Figure 3-6. Decorin- and GAG-binding levels vary among DbpA alleles. Radiolabeled B314 spirochetes harboring the indicated plasmid were added to wells coated with increasing concentrations of decorin or dermatan sulfate, and the percent of bacteria stably bound was determined. (Asterisk indicates that the experiments for B314 expressing $DbpA_{B31}$ were performed in a separate experiment.) Each point represents the mean of four independent determinations.

DbpA was originally identified by its ability to bind to decorin, a dermatan sulfate proteoglycan (66). To test whether allelic variation of DbpA in binding to decorin correlates with binding to dermatan sulfate, the B314 recombinants expressing different DbpA alleles were similarly tested for the ability to bind to increasing concentrations of immobilized decorin. As above, B314 expressing $DbpA_{PBr}$ bound most efficiently to decorin, and $DbpA_{297}$ and $DbpA_{B31}$ promoted attachment to decorin more efficiently than $DbpA_{B356}$ or $DbpA_{N40\text{-}D10/E9}$ (Fig. 3-6 left panel). Notably, $DbpA_{VS461}$ promoted relatively efficient binding to decorin, in spite of promoting only low level binding to dermatan sulfate and epithelial cells (compare to Fig. 3-6, left panel and right panel, and Fig. 3-4). These results indicate that DbpA-mediated bacterial attachment to decorin and dermatan

sulfate are distinguishable, with binding to purified dermatan sulfate correlating better with 293 epithelial cell binding.

The C-terminus of $DbpA_{VS461}$ is required for spirochetal attachment to mammalian cells and to purified decorin or dermatan sulfate.

Basic amino acids are often critical for the ability of GAG-binding proteins to bind their substrate (73), and three lysine residues of $_{DbpA297}$, K82, K163 and K170, were previously identified by chemical modification and mutagenesis as critical for decorin binding (26). In addition, synthetic $DbpA_{297}$ peptides encompassing either K82 or both K163 and K170 directly bound to decorin (115). Höök and coworkers previously showed that the peptides encompassing K82 and corresponding to diverse DbpA alleles were each capable of inhibiting binding of the parental DbpA allele to decorin, implicating this region in decorin binding by diverse alleles. In addition, the sequences flanking K82 of $DbpA_{B31}$ or $DbpA_{N40}$, which differed significantly in their ability to promote binding to epithelial cells, dermatan sulfate and decorin (Fig. 3-3 and 3-4), are nearly identical (Fig. 3-7), suggesting that other segments of the proteins may contribute to GAG, decorin and cell binding. We assessed the potential function of the C-terminus of a *B. afzelii* strain by deleting the $DbpA_{VS461}$ C-terminal 11 amino acids, which encompasses K170 and three other basic residues (Fig. 3-8).

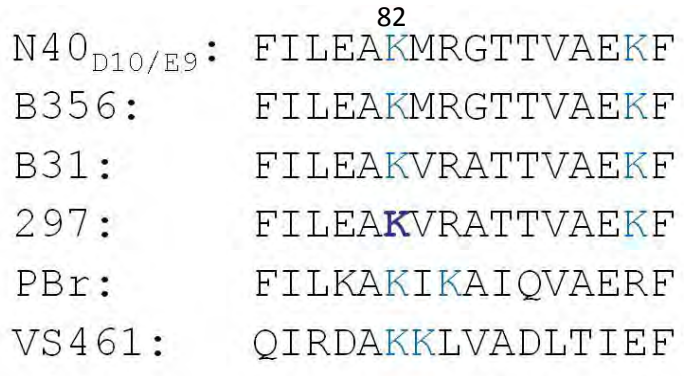


Figure 3-7. Amino acid sequence alignment of the decorin binding regions of the *dbpA* alleles used in this study. Blue font indicates basic amino acids. Bolded lysine indicates critical residue for decorin binding in $DbpA_{297}$.

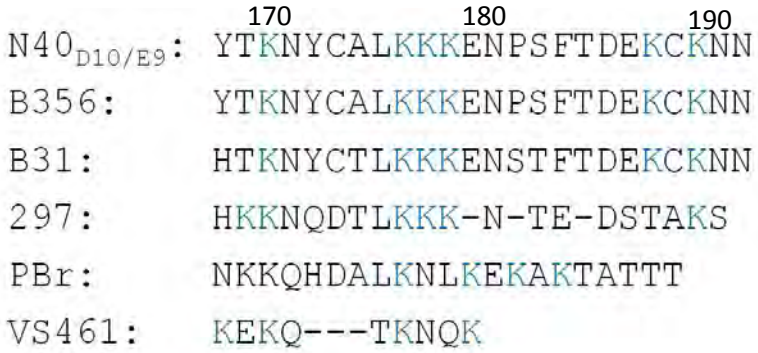


Figure 3-8. Amino acid alignment of the C-terminal regions of the *dbpA* alleles used in this study. Blue font color indicates basic amino acid residues.

We found that deletion of the C-terminal region did not alter surface localization of DbpA, but abolished its ability to promote binding to 293 cells, dermatan sulfate and decorin (Fig. 3-9, "ΔC11"). A fortuitously generated mutant that resulted in the replacement of the C-terminal 11 residues with an unrelated 13-residue sequence also did not promote detectable binding to any of the DbpA substrates (Fig. 3-8, "ΔC11+13"). These results are consistent with the previous implication of C-terminal lysines in decorin binding, and suggest that the C-terminal region is critical for the dermatan sulfate and 293 cell binding by DbpA.

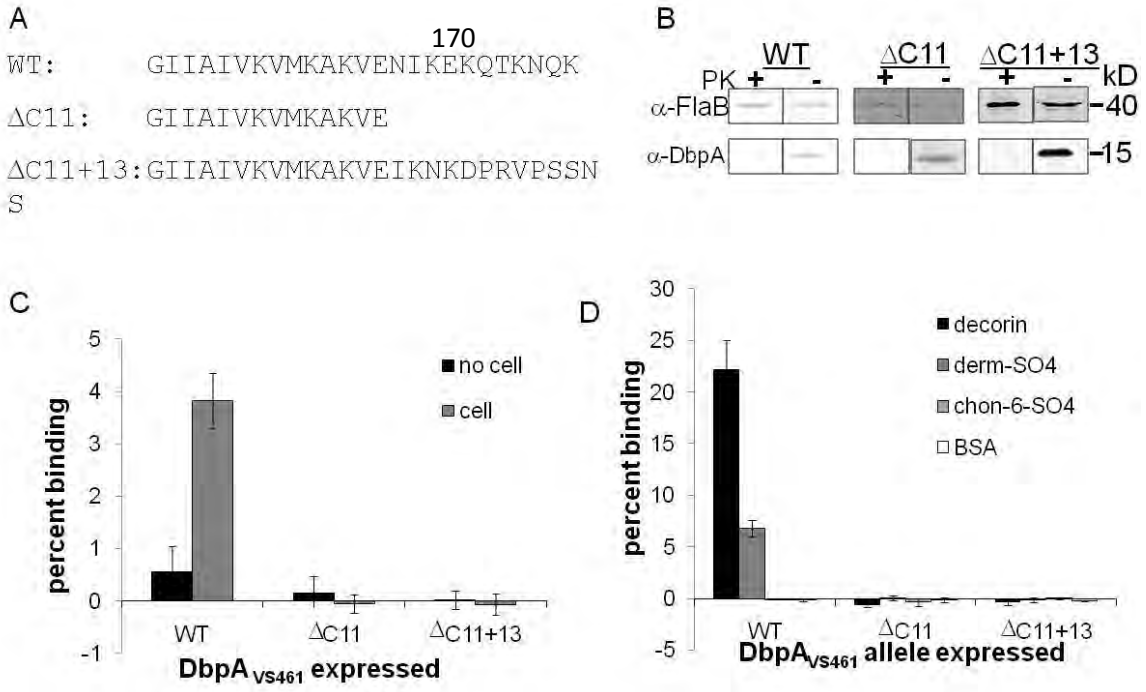


Figure 3-9. The C-terminal region of $DbpA_{VS461}$ is required for binding to mammalian cells and to purified GAGs.

A) The C-terminal amino acid sequences of WT, truncated, and subsitituted $DbpA_{VS461}$. B) WT and mutant $DbpA_{VS461}$are expressed on the surface of strain B314. C) Radiolabeled B314 expressing the indicated forms of $DbpA_{VS461}$ were added to wells with 293 epithelial cells, or D) dermatan sulfate, decorin, chondroitin-6-sulfate, or BSA. The percent of bacteria bound was determined by scintillation count. Each bar represents the mean of four independent determinations ± SD with background binding subtracted.

Discussion

The bacterial factors that contribute to the ability of some Lyme disease spirochete strains to cause disseminated infection are not fully characterized, and may include surface proteins that mediate attachment to mammalian cells or ECM in target tissues. The Lyme disease spirochete exhibits strain-specific variation in GAG-binding specificity that corresponds to variation in the mammalian cell types that are recognized *in vitro* (113). *B. burgdorferi* encodes multiple GAG-binding proteins, including DbpA and B (57), Bgp (112), and BBK32 (56), and the *in vitro* expression pattern of at least some of these adhesins varies with strain (N. Parveen, personal communication). In addition, *B. burgdorferi* might encode GAG-binding adhesins that exhibit strain variation in GAG recognition. DbpA has long been known to be highly variable among Lyme disease spirochetes (123), and could contribute to such strain specific variation.

After confirming that different recombinant DbpA alleles possessed GAG-binding activity, we utilized an *ospC* promoter-based vector to express different DbpA alleles on the surface of the high passage, noninfectious strain *B. burgdorferi* B314, which lacks the *dbpA*-encoding Lp54, as well as any other linear plasmid (compare Fig. 3-10, top panel versus bottom panel).

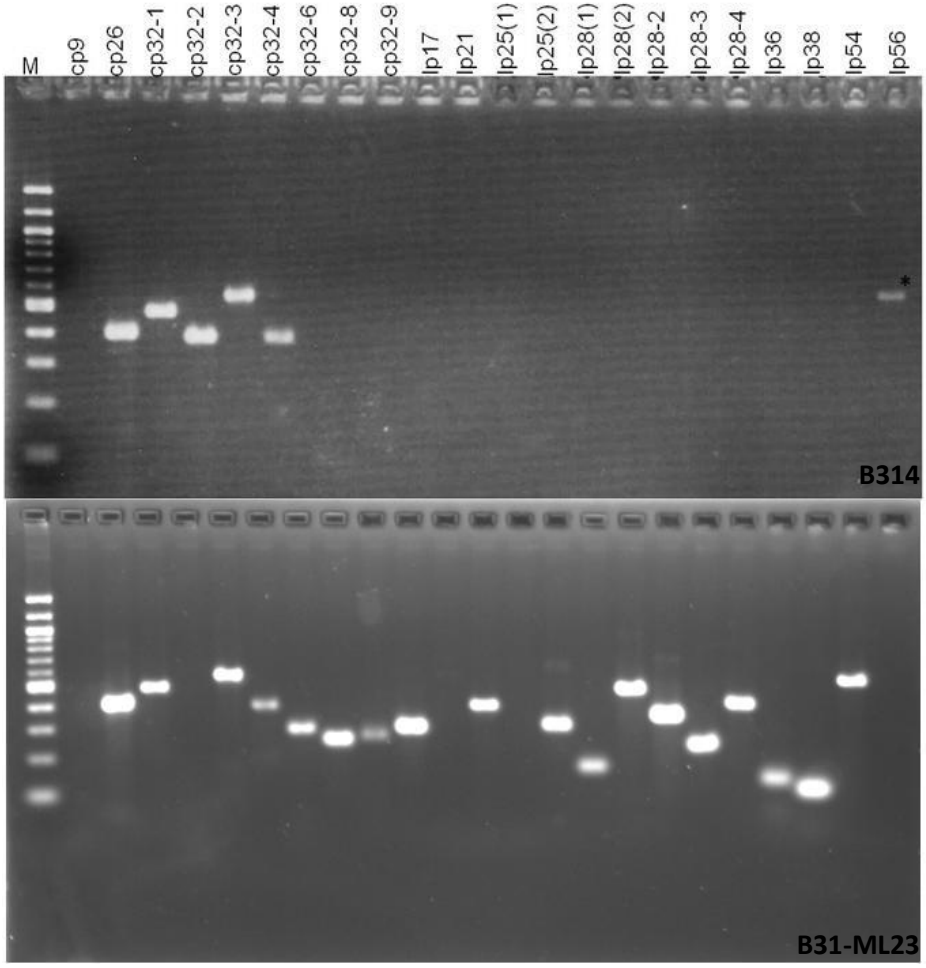


Figure 3-10. Plasmid profile of *B. burgdorferi* strain B314. The non-infectious and nonadherent strain B314 was subjected to PCR using primer pairs that amplify segments of each endogenous plasmid, as described previously (120). As a control to test for the ability of each primer pair to detect the corresponding endogenous plasmid, the infectious strain B31-ML23, which contains all of the endogenous plasmids was analyzed in parallel (plasmids not present are a feature of this clone due to *in vitro* passage) (159). Asterisk denotes that this band denotes that this faint band is not expected size (600bp). M= 100bp ladder.

Given that strain B314 is not known to adhere to any cell line, ectopic expression of surface proteins in this strain provides means to analyze the binding properties of an adhesin in the absence of confounding alternate binding pathways. The set of the DbpA alleles analyzed included representatives of each of the three major species of Lyme

disease spirochete, as well as representatives of *B. burgdorferi* sensu stricto that were associated with different abilities to disseminate in the mammalian host. For each allele, ectopic expression resulted in the ability of the spirochete to bind to decorin, and to epithelial (but not endothelial or glial) cell monolayers, well above background levels. Each DbpA-expressing B314 derivative recognized purified dermatan sulfate, and in all cases, DbpA-mediated epithelial cell attachment was eliminated upon enzymatic removal of this class of GAGs. Thus, a common property of diverse DbpA alleles is the ability to promote epithelial cell attachment through the recognition of dermatan sulfate GAGs.

Although we detected this common binding activity for each DbpA allele, we also observed allelic differences in the level of adhesiveness. For example, $DbpA_{PBr}$ promoted greater than four-fold more efficient epithelial cell attachment than $DbpA_{VS461}$, and more than 20-fold more efficient dermatan sulfate binding. These differences were consistent from experiment to experiment and were a property of the allele expressed, because multiple clones of a given allele were associated with similar binding activities. The differences in binding could not be attributed to the levels of expression of different alleles. First, no dramatic differences in expression levels were detected upon immunoblotting of outer membrane preparations with pooled antisera that collectively recognize the entire collection of DbpA alleles. Second, to the degree that SDS-PAGE and Coomassie blue staining revealed minor potential differences in DbpA levels (Fig. 3-11), we found no correlation between expression level and substrate binding.

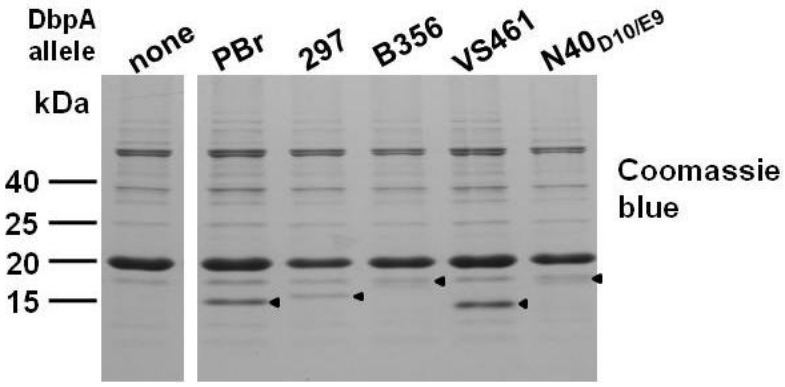


Figure 3-11. Expression pattern of DbpA on the surface of *B. burgdorferi* strain B314. 15 μg of total protein from the detergent phase of Triton X-114 extractions of *B. burgdorferi* strain B314 expressing the indicated DbpA allele were subjected to 15% SDS-PAGE and stained with Coomassie blue stain. Arrows correspond to the DbpA protein expressed.

For example, $DbpA_{PBr}$ and $DbpA_{VS461}$, which as noted above differed dramatically in cell and GAG binding, were expressed at roughly equivalent levels.

As predicted, highly homologous alleles displayed similar binding activities. For example, the dermatan sulfate, decorin and 293 cell binding profiles of $DbpA_{N40\text{-}D10/E9}$ and $DbpA_{B356}$, which are 99% identical in sequence, were virtually indistinguishable. Similarly, $DbpA_{297}$ and $DbpA_{B31}$, which are 90% identical, also mediated roughly equivalent levels of cell, decorin and dermatan sulfate binding. Consistent with the previously documented sequence diversity of DbpA alleles, we observed considerable intraspecies variation in adhesive activities: DbpA alleles derived from *B. burgdorferi* sensu stricto that varied considerably in sequence, e.g., $DbpA_{297}$ and $DbpA_{N40\text{-}D10/E9}$, also varied in attachment activities. Interestingly, given that strain $N40_{D10/E9}$ causes disseminated infection in mice (38), the observation that $DbpA_{N40\text{-}D10/E9}$ promoted levels of adhesiveness lower than most other alleles indicates that robust DbpA-mediated *in*

vitro adhesiveness is not a prerequisite for disseminated infection. Related to this, $DbpA_{N40\text{-}D10/E9}$ and $DbpA_{B356}$ displayed similar binding activities in spite of the fact that *B. burgdorferi* $N40_{D10/E9}$ and B356 differ considerably in their ability to disseminate in mice (38, 155).

Decorin contains a chondroitin or dermatan sulfate GAG chain that is critical for recognition by DbpA (67), and for five of the six DbpA alleles analyzed here, we observed a strong correlation between decorin and dermatan sulfate binding. However, one allele, $DbpA_{VS461}$, promoted relatively high level spirochetal attachment to decorin but low level binding to purified dermatan sulfate. Therefore, although the dermatan sulfate GAG chain of decorin is critical for recognition by DbpA (67), dermatan sulfate and decorin binding are nonidentical. Consistent with this observation, the protein core of decorin was previously shown to be required for detectable binding by DbpA in a radiometric assay (67). Here we found that $DbpA_{VS461}$ displayed a relatively poor activity in promoting 293 cell attachment, suggesting that at least in this instance, dermatan sulfate binding correlates with cell attachment more closely than decorin binding. Definitive determination of the relative contribution of decorin and dermatan sulfate binding in cell attachment mediated by DbpA is complicated by the close structural relationship of these ligands, and awaits further study.

Previous work showed that lysine residues K163 and K170 in the C-terminus of $DbpA_{297}$, along with the centrally located lysine residue K82, were required for binding to decorin (26, 115). The sequence of the DbpA C-terminus is highly divergent, and in

particular the $DbpA_{VS461}$ C-terminus is quite dissimilar from that of $DbpA_{297}$ (123). Nevertheless, the C-termini of all of the alleles tested are highly basic, and thus might contribute to GAG binding. We found that the 11-residue C-terminal segment of VS461, which encompasses K170, is required for the ability of $DbpA_{VS461}$ to promote spirochetal binding to cells, decorin and dermatan sulfate. This and other relatively variable regions of DbpA might contribute to the allelic diversity of attachment activity.

B. burgdorferi mutants that lack DbpA demonstrate a colonization defect in mice (137, 159). In the current study, we showed that the adhesive activity of DbpA is subject to considerable allelic variation. A detailed understanding of the molecular basis of this variation, combined with experimental infection by isogenic infectious strains that express different alleles of DbpA, may provide insight into the adhesive activities of this protein that contribute to infectivity and colonization in the mammalian host.

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CHAPTER IV

Discussion

Host cell attachment is an important step for many pathogens in the establishment of infection and in their pathogenesis. *Borrelia* species are the causative agents of a wide variety of diseases in mammals, including the human diseases Lyme disease and relapsing fever. *Borrelia burgdorferi* sensu lato, the agents of Lyme disease, are capable of colonizing a variety of tissues including the tick midgut, the joints, heart, skin and neural tissue (142). The differences in preferential tissue colonization between species of Lyme disease spirochetes have been well documented (53, 156, 163), and may contribute to the variable chronic symptoms seen in some Lyme disease patients. In Europe and Asia, for example, Lyme disease is more commonly caused by *B. afzelii* and *B. garinii*. These species can cause chronic skin lesions and neurological complications respectively in untreated patients (141). In contrast, *B. burgdorferi* is the predominant strain which causes Lyme disease in the United States and is associated with chronic arthritis in untreated patients. The mechanism(s) of tissue tropism by Lyme disease spirochetes it is currently unknown. Relapsing fever spirochetes such as *B. hermsii* are mainly found in the bloodstream of the infected host, and do not cause a chronic infection in healthy individuals. The most common clinical findings in patients diagnosed with this disease are thrombocytopenia and anemia (22). Relapsing fever Borreliae have been shown to bind to platelets and red blood cells (6, 68), although it is not understood how this interaction contributes to these symptoms.

In our study, we sought to examine the influence of the genetic background of the host on the outcome *B. hermsii* infection. By daily monitoring of infected *rag2*$^{-/-}$ mice, we found that the innate immune response is under at least partial genetic control. In

summary, we found that C57BL/6 *rag2*$^{-/-}$ mice were better able to control the infection than either Balb/C *rag2*$^{-/-}$ or C3H/HeN *rag2*$^{-/-}$ (Fig. 2-2, day 3) and male *rag2*$^{-/-}$ mice were more susceptible than females (Fig 2-4). (For a more detailed discussion, see Chapter 2, pages 41-45). A surprising result arose when infection of C57BL/6 *rag2*$^{-/-}$ mice was compared with its immunocompetent counterpart. Of the immunocompetent mouse strains surveyed, the most sensitive strain was C57BL/6 at day 3 post infection (Fig 2-1). In contrast, when *rag2*$^{-/-}$ mice in this background were examined, this group was the most resistant throughout the course of infection (compare Fig.2-1 and Fig. 2-2). The C57BL/6 mice used in our study were the Bailey substrain (C57BL/6 ByJ), which has a mutation in the interferon regulatory factor 3 (IRF3) gene, while the C57BL/6 *rag2*$^{-/-}$ mice do not. IRF3 is a transcription factor involved in toll-like receptor 4 (TLR4) signaling (61, 106), and one of three regulators of IFNβ that has been shown to be stimulated during viral infection (151). The mutation in the *irf3* gene in C57BL/6 ByJ mice was shown to lead to a defect in IFNβ, which lead to increased sensitivity to *Listeria monocytogenes* infection (61). Our results suggest that IRF3 may play a role in resistance to *B. hermsii* infection, and it would be interesting to compare infection in C57BL/6 and C57BL/6 ByJ mice in order to more thoroughly investigate this. In addition, analysis of an IRF3/rag2 double knockout mouse would be useful in order to examine the role of IRF3 in *B. hermsii* infection in the absence of adaptive immunity.

This study was the first of its kind analyzing the role of strain background of the murine host in the outcome of relapsing fever. Although we found that the innate immune response to *B. hermsii* infection is greatly influenced by the strain background,

and that an IFN response may be involved, we still do not know the specific innate immune mechanism(s) that are involved in resistance to this pathogen. Analysis of blood chemistry panels of infected mice from different genetic backgrounds, as well as analysis of infection of IFNβ knockout mice would be useful in analyzing whether other cytokines are involved in response to relapsing fever infection. In addition, *Borrelia hermsii* has been shown to evade complement killing by binding factor H, which inactivates the opsonin C3b (44, 132, 161). The classical complement pathway requires an antibody response, and therefore would not be triggered in *rag*$^{-/-}$ mice. The alternative complement pathway is activated independently of antibodies and involves the cleavage of C3 to C3b which is then coats the surface of invading pathogens (161). C3$^{-/-}$ mice did not show any differences in the course of infection compared to wild-type mice (161), however, the role of C3 in the absence of an antibody response was not analyzed. A simple assay to test whether genetic background may affect complement mediated killing of *B. hermsii* in the absence of antibody would be to perform serum killing assays using serum from *rag2*$^{-/-}$ mice of different genetic backgrounds. Although it is possible that the ability to bind factor H may confer on *B. hermsii* uniform resistance to serum, one can speculate that some mice, such as C57BL/6 *rag2*$^{-/-}$, might have higher levels of C3 that could promote killing. An additional study would be to generate and analyze infection in C3/*rag2* double knockout mice, which would be expected to be hypersensitive to relapsing fever infection in the absence of both complement- and antibody- mediated clearance of *B. hermsii*.

The most striking findings from our study resulted from the histological analysis of tissue from infected mice which gave insight into the role of host cell attachment by *B. hermsii* in the clearance of this pathogen by macrophages. *B.hermsii* causes thrombocytopenia and anemia in human patients as well as in mice (6, 62). Although it has been shown that thrombocytopenic episodes coincide with peaks in bacteremia (5), the mechanisms that lead to the clearance of blood cells by *B. hermsii* during afebrile periods are not well understood. In our study, we performed histological analyses of the liver and spleen in order to track the course of *B. hermsii* infection in *rag2*$^{-/-}$ mice. The liver and spleen are known to play a role in the clearance of many pathogens, and Sambri *et al*. showed that the liver plays a role in the elimination of both *B. hermsii* and *B. burgdorferi* from the bloodstream (130). In their study, the authors evaluated perfused rat livers for the ability to uptake and kill various radiolabeled Lyme and relapsing fever spirochetes. When infused with radiolabeled bacteria, rat livers were able to rapidly uptake all *Borrelia* species examined, although the efficiency did vary between species (130). Interestingly, no relapsing fever or Lyme disease spirochetes were recovered from the livers by culturing in BSK II medium indicating that the bacteria were killed by the liver. In our study, we examined hematoxylin/eosin stained liver sections and saw evidence of extensive erythrophagocytosis by Kupffer cells (Fig. 2-5A). Although *B. hermsii* cannot be identified by H&E stain, and thus it is not certain that the red-blood cells seen in Kupffer cells were bound to spirochetes, both *B. hermsii* and *B. crocidurae*, another relapsing fever spirochete, have been shown to bind RBC's (30, 68) indicating it is indeed possible that the RBCs visualized within Kupffer cells were bound by *B.*

hermsii. Using fluorescent in situ hybridization (FISH), spirochetes were found to be associated with RBCs in the liver, spleen and heart (Fig. 2-6). In the liver samples, Kupffer cells could not be identified by FISH, and therefore we cannot be certain that the RBC's associated with *B. hermsii* identified in this manner are located within these macrophages. However, our data suggest that spirochete-associated RBCs may be cleared from the bloodstream of mice by uptake in Kupffer cells. Sambri *et al*. could not recover *B. burgdorferi* by culture from infected Kupffer cells in their study suggesting that these macrophages in the liver are capable of uptake and killing of many *Borrelia* species (130). Although the authors did not examine *B. hermsii* interaction with isolated Kupffer cells, these data suggest that the liver does indeed play a role in elimination of *B. hermsii* infections.

More recent studies have shown that Kupffer cells play a role in the immune response to *B. burgdorferi* and spirochetal infections. Marangoni and colleagues demonstrated that in addition to *B. garinii*, *L. interrogans* and *T. pallidum* were able to induce TNFα production in isolated rat Kupffer cells. Interestingly, Polymyxin B which binds to LPS, reduced cytokine stimulation by *L. interrogans* by 50%, but had a negligible effect on *B. garinii* and *T. pallidum* both of which lack LPS. These data indicate that a surface component specific to spirochetes may be responsible for activating cytokine production in Kupffer cells (95). Lee *et al*. observed direct interactions *in vivo* between *B. burgdorferi* and Kupffer cells in the liver vasculature via intravital microscopy. Kupffer cells effectively trapped and phagocytosed spirochetes, which in turn activated *i*NKT cells leading to IFNγ production (83). This study by Lee *et*

al. confirms the findings by Sambri *et al*. and demonstrates that Kupffer cells can in fact eliminate spirochetes from the bloodstream. These data provide insight into mechanism of immune clearance of spirochetal pathogens. However, to date no studies have provided evidence of a direct interaction between *B. hermsii* and Kupffer cells. Our data suggest that such an interaction does indeed occur, and may be influenced by the genetic background of the mouse. Although we did not see qualitative differences in the histopathology between tissues of *rag2*$^{-/-}$ mouse strains, C57BL/6 *rag2*$^{-/-}$ mice were able control bacteremia more effectively than either Balb/C *rag2*$^{-/-}$ and C3H *rag2*$^{-/-}$. Based on these data, it would be of interest to perform a similar study with Kupffer cells isolated from mice of different backgrounds. A study of this kind could provide insight into mechanisms of clearance of *B. hermsii* infection and whether attachment to RBC's plays a role in the clearance of this pathogen. In sharp contrast to relapsing fever spirochetes, cell adhesion appears to play a very different role in Lyme disease pathogenesis in which spirochetes appear to use cell adhesion to establish and maintain infection.

Lyme disease is a multifaceted disease caused by spirochetes of the genus *Borrelia*. In order to maintain enzoonotic its life cycle, Lyme disease Borreliae express numerous adhesins which allow colonization of the tick vector and subsequent colonization of mammalian host (89, 108, 168). In order to establish and maintain infection, Lyme disease spirochetes maintain tight regulation of their surface adhesins, each which bind one or more cellular or extracellular matrix component (69, 76, 107, 121). Different Lyme disease genospecies have been shown to preferentially colonize

different tissues in the mammalian host. However, it is not know these adhesin(s) contribute to the tissue tropism seem among various Lyme disease Borreliae.

Some strains of Lyme disease spirochete have the capacity to disseminate from the initial site of infection via the bloodstream and colonize other tissues, including skin, heart, joint, and nervous tissue. In addition, Lyme disease Borreliae have been shown to bind to multiple different cell types *in vitro*. These spirochetes express a plethora of adhesins, for which some the corresponding ligand(s) have been identified. The multitude of adhesins may reflect the variety of tissue and cell types that Lyme disease Borreliae are known to bind to. Consistent with this, DbpA promotes binding to epithelial cells, whereas the related adhesin DbpB promotes binding not only to epithelial but also to glial cells. Additionally, different adhesins may play a role at different stages of extravasation. A study by Norman *et al*. demonstrated that dissemination of *B. burgdorferi* through the vasculature of infected mice involved different stages which the authors termed tethering, dragging, stationary adhesion and extravasation. This is reminiscent of leukocyte extravasation from the vasculature, which occurs in multiple steps, each mediated by different molecules (110, 126). For example, the cell adhesion molecules L- and P-selectin mediates rolling adhesion, while stationary adhesion is mediated by β2-integrins (31, 88). For *B. burgdorferi*, it was shown that BBK32 mediated GAG and fibronectin binding were required for the various spirochete interactions with the vasculature (99, 104). One can speculate then that multiple adhesins are required for invasion and colonization of tissues such as the heart. As with adherence

to and escape from the vasculature, colonization may also involve multiple steps such each of which could require a different adhesin to recognize different host receptors.

In this study, we expressed alleles of the highly heterogeneous surface lipoprotein DbpA from six Lyme disease spirochetes on the surface of the non-adherent, non-infectious *B. burgdorferi* strain B314. *B. burgdorferi*, *B. garinii*, and *B. afzelii* are each associated with different chronic Lyme disease manifestations in humans (82, 150), and the collection used in this study included representatives from each of the three species (Table 3-3). In summary, we found that all *dbpA* alleles tested conferred varying ability to bind epithelial cells, decorin, and dermatan sulfate to strain B314 (see chapter 3, pages 76-81 for a detailed discussion on these results). Based on our data, we made three important observations. The first observation was that $DbpA_{PBr}$ appears to be in a class alone, showing the strongest binding phenotype to all binding to all substrates tested. $DbpA_{PBr}$ is, however, the only *B. garinii* allele tested in this study. Analysis of DbpA alleles from additional strains of *B. garinii* for similar *in vitro* binding phenotypes may provide insight as to whether strong binding to decorin and/or dermatan sulfate is required to cause the neurological symptoms associated with this species. Second, DbpA from *B. afzelii* strain VS461 bound to decorin more strongly (Fig. 3-5, left panel) than to dermatan sulfate (Fig. 3-5, right panel) or epithelial cells (Fig. 3-4). The decorin binding domain of DbpA has been identified (115). While it is currently unknown whether a specific domain of DbpA mediates GAG binding, these observations lead us to speculate that the two binding activities of DbpA may be separable. However, we only tested the DbpA allele from one *B. afzelii* strain and it would be of interest to additional alleles

other *B. afzelii* strains which may provide insight in identifying the GAG binding domain of DbpA. Third, the alleles $DbpA_{N40}$ and $DbpA_{B356}$ which share 99% homology have identical *in vitro* binding phenotypes. It is interesting to note that while $DbpA_{N40}$ does not bind to epithelial cells, decorin or dermatan sulfate at very high levels, *B. burgdorferi* strain N40 is very invasive in the mouse model (84), leading us to hypothesize that robust DbpA mediated binding may not be required for invasive disease. In contrast, strain B356 does not cause invasive disease (155) which indicates that the *in vitro* binding phenotypes seen in our assay may not reflect disease phenotype *in vivo*. The possibility exists that some DbpA alleles may recognize an as yet unidentified cell surface or ECM molecule which could contribute to the variation in binding levels by the different DbpA alleles used in this study. This phenomenon could be explored in a future study by including a greater number of different cell types which express different GAG types and ECM molecules in our binding assays. To our knowledge, there are no studies to date which evaluate how genetic variation of a specific Borrelial lipoprotein contributes to Lyme disease pathogenesis. However, were able to show a specific interaction between all DbpA alleles examined with the GAG dermatan sulfate (Tables 3-4 and 3-5). Our assays provided a starting point to evaluate the differences in the binding capabilities of DbpA alleles from different Lyme disease genospecies. However, given the disparity noted between the *in vitro* binding properties of $DbpA_{N40}$ and $DbpA_{B356}$ and the invasive properties of their associated *B. burgdorferi* strains, we will use a method to evaluate how different DbpA alleles may contribute to disease *in vivo*.

B. burgdorferi strain B314 is noninfectious and possesses only four circular plasmids and no linear plasmids (Fig. 3-10), thus, the strains generated for this study cannot be used in a traditional mouse infection model. Thus, in order to evaluate the *in vivo* contribution of different DbpA alleles in the pathogenesis of Lyme disease, we will complement a *dbpBA* null strain generated in the laboratory of Dr. Jon Skare (159). The strain generated by Dr. Skare's lab lacks lp25 which contains the *pncA* gene, which is required for mammalian infection by *B. burgdorferi* (119). We will therefore use a shuttle vector containing *pncA* to express each *dbpA* gene (119). In the current study, we used a shuttle vector containing the *ospC* promoter which drives very high level expression of DbpA. For this future study, because we are evaluating DbpA in an infectious strain, we will use the native *dbpA* promoter to express each allele in order to achieve wild-type like expression levels. In their study, Weening *et al*. showed that their *dbpBA* null was not infectious and that complementation restored infectivity to near wild-type levels (159). In our study, because we are complementing with dbpA alone, the ID_{50} of our complemented strains are expected to be higher than that of the complemented strain used by Weening/Skare (~300 spirochetes) which was complemented with both DbpA and B. We will therefore use an infectious dose of 10^4 *Borrelia* per infection. Although this dose is much higher than the ID_{50} of the parent strain (~150), it is lower than that of the null strain (>10^6) and also lower than the dose used by Shi *et al*. (10^5) where no difference in infectivity was seen between their *dbpBA* null strain and its parent (138). As with strain B314, we expect that different *dbpA* alleles will have varying abilities to complement the *dbpBA* null strain which will be evaluated by extracting DNA

from various tissues from infected mice and measuring spirochetal load by quantitative real-time PCR. One caveat to the design of this experiment is that the complemented strain is derived from a wild-type infectious strain, and as such we are evaluating the *in vivo* properties of DbpA in the presence of other adhesins. For example, *B. burgdorferi* strain B356 and *B. afzelii* strain VS461 have been shown to cause a localized skin infection (141, 155). We therefore expect that the *dbpBA* null strain complemented with DbpA from either of these strain to localize to the skin of an infected mouse. However, *B. burgdorferi* expresses multiple adhesins on its surface including Bgp (112) and BBK32 (118) which also bind to GAGs and may have redundant functions which could possibly lead to a lack of differences between the DbpA alleles we are analyzing.

In order circumvent the problem of evaluating the *in vivo* properties of different DbpA alleles in the presence of other adhesins, it is possible to use the strains generated in this study in a murine infection model using a high dose (~10^9) infection. Although B314 is non-infectious, it possible to use a high dose, short duration infection and measure spirochetal DNA in tissue. Additionally several studies have used intravital microscopy to evaluate borrelial interactions with murine vasculature in various tissues (83, 104) which could be used to evaluate strain B314 expressing different DbpA alleles.

In summary, we analyzed the role of host-cell interactions in the pathogensis of pathogenic Borreliae, *Borrelia burgdorferi* sensu lato and *Borrelia hermsii*. In the case of the former, evidence in our study suggests that attachment to host cells is a critical step in the establishment of Lyme disease, is required for tissue colonization which can lead to

a plethora of chronic symptoms if left untreated. In contrast, in the case of infection by *B. hermsii* we found evidence which suggests that attachment to red blood cells may lead to clearance of this pathogen from the bloodstream via phagocytosis in the liver. Our studies provide a starting point for further research in these areas.

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